New ways to optimize breast cancer treatment

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General introduction

General introduction

In this thesis ways for optimizing breast cancer treatment, and aspects of early systemic disease in particular, are described. Breast cancer is the most frequent type of cancer in women in the Netherlands, as one in ten women will develop breast cancer during life time. The concept of breast cancer as a systemic illness has been increasingly translated into systemic treatment over the past decades. Early systemic tumor spread in breast cancer patients is presumed to be commonly present at the first time that patients present. This is supported by the fact that following effective local treatment, many patients manifest metastatic involvement over time and that improvements in local control have been shown to provide only a small decrease in distant metastases (1, 2). Early ('adjuvant') systemic treatment in concert with local therapy, was shown to have a beneficial impact on survival rates of breast cancer patients (3), also when analyzed separately from the benefits induced by diagnostic screening (4).

Clinical trials in recent years have aimed at further optimization of early treatment modalities by means of chemotherapy, hormonal or biological approaches (5). Dose-intensification of chemotherapy to optimize adjuvant treatment, gained interest in view of the validation of this approach in the advanced breast cancer setting (6). The possible benefits of high-dose chemotherapy and stem cell transplantation in the adjuvant setting for breast cancer have been studied in a number of large randomized studies, the results of which are expected to be published soon (7). The selection of breast cancer patients for adjuvant treatment, is traditionally based on the evaluation of tumor presence in the axillary lymph-nodes. However, it is clear that conventional evaluation of axillary lymph node status has its flaws. Forty percent of women with tumor positive lymph nodes survive more than 10 years, and vice versa: distant metastases develop in 20 to 30 % of patients with node-negative cancer (8). The development of those distant

metastases suggests that there may be alternative metastatic routes, other than the classical sequence of tumor-lymph node-hematogenous metastases (reflected in the TNM classification for breast cancer staging, Union International Contre le Cancer 1997). Support for this concept was recently provided in a study by Braun et al. (9), indicating that the ability of tumor cells to expand hematogenously to the bone marrow is independent of their ability to metastasize to axillary lymph nodes. Although primary tumor characteristics may be helpful for selecting the group of node-negative patients at risk for metastases (10), the identification of new markers which predict relevance of early adjuvant systemic treatment has gained much interest in the last decade. Particularly in view of the use of adjuvant high-dose treatment, requiring haematopoietic stem cell transplantation to counteract profound bone marrow aplasia, this selection may have clinical impact. In addition, detection of tumor cells in hematopoietic stem cells products can provide information on their clinical impact. It is possible that tumor cell contamination of haematopoietic stem cells may directly contribute to relapse in breast cancer patients, as was described previously in haematological malignancies (11). However, in the adjuvant breast cancer treatment setting, the impact of tumor cells in stem cell products is as yet unknown (11).

Thus, sensitive staging techniques for the detection of micrometastatic disease may be clinically helpful in the treatment of breast cancer. It could serve as a tool for direct evaluation of the effect of early systemic treatment. Also, it may prove helpful for the selection of patients for early treatment modalities. Furthermore, if minimal amounts of tumor cells would prove detectable, (*in vitro*) methods for removing these tumor cells could be evaluated. These data provide the basis for this thesis: the evaluation of aspects of breast cancer, and the detection and removal of early systemic disease in particular, thus possibly providing tools for optimizing treatment.

Content of the thesis

In chapter two an overview is given concerning methods for detecting minimal amounts of tumor cells in peripheral blood, peripheral blood stem cells and bone marrow. In this respect, the possible role of mobilization of tumor cells by means of hematopoietic growth factors is described. Subsequently, the various options for removing these tumor cells are discussed.

In chapters three, four and five, the detection of tumor cells based on the presence of the expression of epithelial glycoprotein-2, EGP-2. As a pan-carcinoma marker, EGP-2 is universally expressed in breast cancer specimens (12). As such, EGP-2 is a commonly used target antigen in a number of carcinoma-directed immunotherapeutical approaches (12- 14). In chapter three, molecular detection of the presence of EGP-2 by means of a quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) method is validated and compared to immunohisto(cyto)chemical detection by means of the MOC31 antibody, directed against EGP-2. EGP-2 was found to be expressed at different levels in primary tumor samples. To make assumptions regarding the quantification of tumor load in the blood stream of patients, its appears necessary to use EGP-2 expression in both primary tumor and blood, in individual patients. In chapter four, therefore, the EGP-2 marker is used for detecting tumor cell presence in primary tumor samples, sentinel lymph nodes and peripheral blood samples from breast cancer patients. In this perioperative setting, the ability to detect minimal amounts of tumor cells may serve as a tool for selecting patients for adjuvant treatment. Analysis of EGP-2 expression was performed in an automated way by means of a real-time PCR device (Light Cycler). Due to simplified technical protocols using this machine, the risk of contamination was found to be reduced, and therefore in chapter five, this technique was used for sequential detection of minimal residual tumor cells in the peripheral blood of patients randomized to receive adjuvant standard or high-dose

chemotherapy and autologous peripheral blood stem cell support. In this setting, the ability to detect minimal amounts of tumor cells may serve as a tool for evaluating the efficacy of these treatment modalities.

In chapters six and seven the options for removal (or 'purging') of minimal amounts of tumor cells are explored. To this end, the bispecific antibody BIS-1 was used. This bispecific antibody, directed against EGP-2 on tumor cells and CD3 on T lymphocytes, creates functional cross-linking of T cells and tumor cells allowing the delivery of a tumor cell specific lethal hit inducing specific epithelial tumor cell kill *in vitro* and *in vivo* (13). In chapter six, a method is described for purging tumor cells from peripheral blood stem cells harvests of breast cancer patients, receiving adjuvant high-dose chemotherapy and autologous peripheral blood stem cell support. T lymphocytes, present in the peripheral blood stem cell harvests, were activated for optimal tumor cell kill potential, and retargeted by BIS-1. In chapter seven, a similar method was then applied to the setting of minimal tumor cell contamination of cryopreserved ovarian tissue, of female cancer patients with impending loss of fertility due to cancer treatment. Tumor cell kill and morphological follicle survival were studied in an *in vitro* model in which activated lymphocytes and BIS-1 were added to tumor cells, in the presence or absence of a suspension of human frozen-thawed ovarian tissue. These studies on the purging of minimal amounts of tumor cells may contribute to the safe auto-transplantation in cancer patients, of either stem cells or ovarian tissue.

In chapters eight to ten, various aspects of breast cancer treatment, assessed in patients (chapter eight), or patients samples (chapters nine and ten) are described. The issue of febrile leukopenia in breast cancer patients receiving intermediate high-dose chemotherapy is described in chapter eight. To reduce the incidence of febrile leukopenia, the use of prophylactic antibiotics was compared to hematopoietic growth factor recombinant human granulocyte-stimulating growth

factor (rhG-CSF). The manageability of chemotherapy induced side-effects may improve the safe treatment of breast cancer patients. Chapter nine focuses on hematopoietic reconstitution and the possible induction of increased aging in hematopoietic stem cells, in breast cancer patients receiving either adjuvant standard or high-dose chemotherapy and autologous peripheral blood stem cell transplantation. Telomere length, as a marker for cell turn-over and therefore of aging, was measured in the nucleated peripheral blood cell fraction of these patients. The increased aging of hematopoietic stem cells due to stem cell transplantation may have important undesirable long-term effects, that could be clinically relevant in patients with a relatively good prognosis. Insight in possible causes for long-term implications of adjuvant treatment are important for optimizing breast cancer treatment. In chapter ten, primary breast tumors are evaluated for the presence of death receptors and ligands. The presence of death receptors Fas (receptor for Fas Ligand, FasL), and DR4 and DR5 (receptors for TNF-related apoptosis inducing ligand, TRAIL) in primary breast tumors, may possibly allow treatment with their ligands. In addition, as death receptors may be up-regulated by estrogen deprivation, these parameters were evaluated in tumors after pre-operative anti-estrogen therapy. The possibility to assess these effects in individual tumors may allow more 'tailor-made' breast cancer treatment in the future.

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The impact of hematopoietic growth factors on supportive care in clinical oncology: with special attention to potential tumor cell contamination in the stem cell harvest

A review

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Hematopoietic growth factors (HGFs) are now for a number of years available for use in oncological patients. Drugs currently registered are: granulocyte colony stimulating factor (G-CSF) and granulocyte macrophage colony stimulating factor (GM-CSF) for neutrophil stimulation and erythropoietin for stimulation of erythropoiesis. With several other HGFs phase I, II and III studies have been performed or are ongoing. The new as yet unregistered compounds are often under investigation for their capacity to stimulate thrombopoiesis.

Indications for the use of neutrophil stimulating growth factor are: 1) prevention of neutropenia and its complications following chemotherapy, 2) treatment of neutropenic fever, 3) prevention or abbreviation of neutropenia during chemotherapy requiring bone marrow reconstitution and 4) peripheral stem cell harvest.

1. The prevention of neutropenia and its complications following chemotherapy

Bacterial and fungal infection is a considerable cause of death in cancer patients (2). Leucopenia due to multi-agent chemotherapy regimens, is associated with substantial febrile morbidity (3, 4). Infection rates increase when the peripheral blood granulocyte count falls below $0.5x10^9$/L, and especially when it is less than $0.1x10^9$/L or when duration of leucopenia is prolonged (5). Haematopoietic toxicity can be decreased by chemotherapy dose reduction. However, this may have a negative effect on treatment outcome. Therefore, other means of reducing and preventing febrile leucopenia have been studied (6). Prophylactic haematopoietic growth factors are used to reduce the incidence of febrile neutropenia, by reducing the duration of neutropenia (1, 7).

In a number of phase III studies in which chemotherapy was used that induced a neutropenic fever of at least 40%, G-CSF was found to reduce the incidence of severe neutropenia, it ameliorated neutrophil nadir, reduced neutropenia duration and

reduced the incidence of neutropenic fever (50%), culture positive infections and the use of antibiotics (1).

Another approach might be the use of chemoprophylaxis by the use of prophylactic antibiotics. Also, prophylactic antibiotics (so called chemoprophylaxis) have shown to reduce the risk of febrile morbidity (8). Various antibiotics have been used for this purpose (9, 10). Chemoprophylaxis by quinolone-based treatment was found to be particularly effective for intestinal decontamination (11). This way, infections with Gram negative bowel organisms, a major cause of morbidity and mortality in the leucopenic patient, can be substantially reduced (12). Next to bacterial infections, fungi also constitute a major problem in neutropenic patients, requiring specific approaches for prevention and therapy. A number of anti-fungal agents, for instance amphotericin B, can be used for chemoprophylaxis (13, 14).

In two studies comparing the use of granulocyte-stimulating growth factor (G-CSF) to G-CSF plus antibiotics (15, 16), an additional, beneficial effect of antibiotics was found. Furthermore, a few placebo-controlled reports on prophylactic norfloxacin or ofloxacin also clearly indicated a positive contribution in this setting (17-19). In a retrospective study, prophylactic G-CSF was compared to prophylactic oral ciprofloxacin in ovarian cancer patients with paclitaxel induced leucopenia (20). No difference between fever rates was observed when prophylactic G-CSF was compared to oral ciprofloxacin, but comparison with a historical control group not receiving any prophylactic agent, showed a clear benefit from prophylaxis in these patients with relatively short but deep leucopenia. In a non-randomized dose-finding study (21), early stage breast cancer patients received 5-fluorouracil (5-FU, 500 mg/m2 intravenously), epirubicin (60 mg/m2 IV) and cyclophosphamide (75 mg/m2 for 14 days). Co-trimoxazol was administered prophylactically to a group of 89 consecutive

patients, and fever rates decreased significantly compared to the control group not receiving prophylaxis. Currently for example in our center a study to compare G-CSF versus quinolones is analyzed. A possible disadvantage of prophylactic quinolones may be the development of resistant organisms (22). Although most data actually contradict this (23), a possible way to circumvent the risk of developing infection in these immunocompromised patients would be to evaluate the efficiency of intestinal decontamination by monitoring of faecal organisms during prophylactic treatment. This way, antibiotic treatment can be altered if necessary to provide the best prophylaxis. Another option would be to limit the use of prophylaxis to those patients who have actually developed grade IV leucopenia. If chemoprophylaxis induces resistance at all, it may be limited by these precautions.

Therefore, chemoprophylaxis can be viewed as a reasonable alternative for G-CSF in preventing febrile leucopenia. However, in future studies placebo-controlled assessment of chemoprophylaxis in high risk patients would be useful.

2. Treatment of neutropenic fever

Randomized trials have not conclusively demonstrated a clinical benefit when G-CSF and GM-CSF are given for uncomplicated febrile neutropenia. The routine use of these factors in this setting can not be recommended. We performed a study to determine whether GM-CSF used in addition to standard inpatient antibiotic therapy shortens the period of hospitalization due to chemotherapy induced neutropenic fever. Patients with a hematologic (n=47) or solid tumor (n=87) who had severe neutropenia and fever were randomly assigned to receive GM-CSF 5 μg/kg/d (n=65) or placebo (n=69) in conjunction with broad spectrum antibiotics for a minimum of 4 days and a maximum of 14 days. GM-CSF/placebo and antibiotics were stopped if the neutrophil count was

greater than $1.0x10^9$/L and temperature less than 37.5°C during 2 consecutive days, or for a leucocyte count ($10x10^9$/L, both followed by a 24 hour observation period (hospitalization period). Compared with placebo, GM-CSF enhanced neutrophil recovery. Median neutrophil counts at day 4 were $2.5x10^9$/L (range, 0-25) in the GM-CSF arm and $1.3x10^9$/L (range 0- 9) in the placebo arm. No significant difference was observed with regard to median number of days with less than $1.0x10^9$/L neutrophils or days of fever. The median number of days patients were hospitalized while on study was comparable in the GM-CSF and placebo groups at 6 (range 3-14) versus 7 (range 4-14). Quality of life scores in 90 patients demonstrated differences in favor of the placebo group. Hospital costs were higher for GM-CSF treated patients if GM-CSF was included in the price. These results indicate that GM-CSF did not affect the number of days for resolution of fever or the hospitalization period for this patient group, although a significant effect of GM-CSF was observed on neutrophil recovery (24).

3. Reduction of neutropenic period during chemotherapy requiring bone marrow reconstitution

Patients receiving high-dose chemotherapy followed by bone marrow or peripheral stem cell reinfusion have a slightly faster neutrophil recovery if stem cell reinfusion is combined with G-CSF or GM-CSF administration (1).

4. Peripheral stem cell harvest

The most important role of CSFs is in the phase of peripheral blood stem cell (PBSC)

mobilization. CSFs facilitate mobilization of hematopoietic stem cells. The advantage of PBSC transplantation following high-dose chemotherapy is that it reduces the duration of not only neutropenia but also thrombocytopenia compared to autologous bone marrow transplantation. It has been shown to shorten the duration of neutropenia and thrombocytopenia, and it reduces incidence of infections and hospital stay. Stem cell harvest before high-dose chemotherapy can also collect tumor cells from the circulation. The exact relevance for the clinic of the presence of tumor cells in the stem cell harvest is as yet unknown. Several purging methods are developed and currently in clinical trial. Ex-vivo culturing of hematopoietic stem cells in the presence of CSFs is an other way to eliminate tumor cells. Therefore, the role of tumor cell contamination is more extensively explained in this review.

4.1 Tumor cell detection: introduction

The increased potential clinical relevance of adjuvant high-dose chemotherapy in solid tumors has raised the relevance of tumor cell contamination in bone marrow. There is increasing evidence that not only in bone marrow but also, although studies do suggest less likely, in peripheral blood stem cell harvest, tumor cell infiltration may be involved (25, 26). Early papers reported on sporadic findings of tumor contamination of solid tumors in the peripheral blood. In other studies blood was collected from cancer patients during or just after surgery and these samples often contained significant numbers of tumor cells, yet these patients do not always develop metastatic disease. Often the clinical follow up in these studies was not long (27). It may be that the circulating tumor cells are not always viable or able to form metastases. In the animal model, however, it is shown that many viable tumor cells are shed into the circulation. So, the significance of presence of tumor cells in the circulation is as yet

undetermined. The process of metastasis involves multiple host-tumor interactions and it is thought that only a few of all the circulating cells are successful in establishing metastatic colonies. Tumor cells are likely to acquire the ability to metastasize as a result of cumulative genetic changes that provide the cells with progressive metastatic capability through alterations in cell regulatory mechanisms, secretion of proteases, induction of angiogenesis, increased cellular motility and altered expression of cell adhesion molecules (28). The ability to detect very small numbers of tumor cells may provide the clinician with an important predictive tool with respect to recurrence and might help in a better selection for adjuvant therapy.

4.2 Bone marrow micrometastases

Recently, with the availability of multiple antibodies directed against epithelial cells, it became clear that in breast carcinoma patients without signs of metastatic disease tumor cells can often be detected in the bone marrow. In breast carcinoma patients without evidence of distant metastases. Redding et al. reported already in 1983 a study in 110 patients (29). They performed immunocytochemical analysis on bone marrow smears and detected tumor cells in 28% of the samples. Bone marrow was positive in 24% of the patients who had no lymph node involvement. In a larger, identical diagnostic group of 285 patients, 27% had positive bone marrow with an antiserum raised against the epithelial membrane antigen EMA (30). In following studies also the effect on prognosis was analyzed. Cote et al. studied 49 patients with stage I and II operable breast cancer (31). With a 30 months median follow up time there was an association between early recurrence and tumor infiltration of the bone marrow. Also in this study, the importance of quantifying the number of cancer cells was shown. Multivariate analysis indicated the ratio cancer cells: nucleated cells was the only

significant variable for the prediction of early recurrence (>10 cells per $4x10^6$ nucleated cells). Diel et al. reported in 1992 on a much larger study. In 260 patients with primary breast carcinoma bone marrow aspirates obtained from six sites of the skeleton were analyzed for tumor cells (32). After density centrifugation, cells in interphase were smeared and stained. For the immunocytologic reaction the monoclonal antibody TAG12 was used. Tumor cells could be detected in 115 (44%) of the bone marrow samples. The presence of tumor cells correlated with tumor stage, nodal stage and tumor grading. Relapses occurred especially in those with positive bone marrow. The highest prediction for distant metastases was obtained in this study by combining the nodal status, negative progesteron receptor and tumor cell presence in the bone marrow. Pantel et al. reported on the immunological detection of various markers associated with tumor progression. In cytokeratin 18 positive bone marrow from patients with cancer from the breast, gastrointestinal tract or the colon, proliferation markers Ki-67 and p120, and erb2 oncogene expression was studied. Only few cells labeled with these markers, but CK18-erb2 double labeling was associated with increased clinical stage in breast and gastrointestinal carcinoma (33). The role of bone marrow metastases in cancer of the breast, lung, stomach and colon have been investigated in various studies (29-32, 34-38). In general, it has been shown that detection of bone marrow metastases predicts for recurrent disease, and that results have correlated with clinicopathological staging parameters. A recent study, in which a large number (n=552) of bone marrow aspirates from stage I, II and III beast cancer patients were analyzed by means of cytokeratin directed antibody A45-B/B3, described micrometastic bone marrow disease, unrelated to the presence or absence of lymph-node metastases (39). Thirty-six percent of all patients showed bone marrow micrometastases, and of patients with node-negative disease this percentage was 33%.

It appears conceivable therefore, that there may be alternative metastatic routes, other than the classical sequence of tumor-lymph node-hematogenous metastases (reflected in the TNM classification for breast cancer staging, Union International Contre le Cancer 1997). The apparent ability of tumor cells to expand hematogenously to the bone marrow is independent of their ability to metastasize to axillary lymph nodes, which indicates that sampling of bone marrow in addition to sampling of axillary nodes could possibly lead to more accurate staging in breast cancer. It should be reminded though, that the percentage of bone marrow micrometastases in this study is higher than the percentage of patients that are at risk for relapse (for instance: in tumors <1 cm: 35% bone marrow micrometastases, whereas long-term survival in these patients is 95%). Therefore, one might suggest that this additional staging method is useful preferably to identify those patients without bone marrow or lymph node metastases as very-low risk patients, who do not require systemic treatment.

4.3 Peripheral blood

The presence of tumor cell contamination in the peripheral blood is considered relevant because peripheral stem cell harvest and reinfusion after high-dose chemotherapy is increasingly thought to be a useful treatment for patients with high risk of tumor relapse. Also, tumor cell contamination in peripheral blood can be considered a potentially useful diagnostic tool, allowing a better selection of patients that may benefit from adjuvant chemotherapy. If a simple blood test could have prognostic value, this might be a clinically very interesting alternative to other staging methods. The detection of single tumor cells in peripheral blood or peripheral blood stem cells (PBSC) is a particular challenge. Techniques to detect these rare circulating cells should be both highly sensitive and specific. The markers used should be

indicators of tumor cell presence in the blood, not expressed by haematopoietic cells, and not shed from the tumor into the circulation. In haematological malignancies, tumor-specific qualities are available for this purpose. Gene translocations, such as t(14;18) in follicular lymphpoma, were found to be accessable for molecular detection, using polymerase chain reaction (PCR) amplification (40). The presence of t(14;18) bearing cells in bone marrow, peripheral blood stem cells and possibly peripheral blood was found to be associated with early recurrences (41-43). Also in other haematological malignancies, specific gene translocation could be detected at a cellular level (44). However, the cytogenetics of solid tumors are considerably more complex and less well defined than those of haematological malignancies. Mutations of either oncogenes or tumor suppressor genes have been studied, but a common problem is the lack of consistency within tumor types, and the number of different mutations. For instance, p53 tumor suppressor gene mutations are found throughout the open reading frame, and although 'hotspots' have been identified, even these extend over four exons. Thus, other targets have been sought. Instead of tumor specific qualities, the detection of tissue specific antigens or enzymes by reverse transcriptase-PCR (RT-PCR) has been evaluated. This approach is based on the fact that malignant cells continue to express specific marker characteristics of their tissue of origin. RT-PCR was found to be an extremely sensitive technique, allowing the detection of one tumor cell in $1x10^6$-$1x10^7$ normal cells (45, 46). Thus, cells expressing prostatic specific antigen (PSA) were found in the peripheral blood of prostatic cancer patients without metastases (46). Tyrosinase expression was used as a marker for melanoma cells, showing a correlation between clinical disease stage and a positive tyrosinase RT-PCR (47). A similar result was obtained in neuroblastoma patients (48). For breast cancer cells, specific epithelial markers were studied. Datta et al. developed a RT-PCR for keratin 19 (K19) transcripts

to identify breast carcinoma cells in the peripheral blood and bone marrow of patients with breast cancer. K19 mRNA is a marker for the intermediate filament protein which is found in all normal and malignant breast cells and also in a number of simple epithelial cells and their malignant counterparts (49). In their experiment the K19 RT-PCR reliably detected 10 breast cancer cells in $1x10^8$ normal peripheral blood mononuclear cells (49). A similar experiment using also keratin filament transcripts, was earlier performed by Traweek et al. (50). In contrast to Datta et al. they detected presence of keratin 19 activity in stromal cells (fibroblasts and endothelial cells). These stromal cells could be a possible source of keratin 19 transcripts.

Immunocytochemistry is also a widely applied technique to detect single tumor cells from solid malignancies. The detection of micrometastases in bone marrow, using antibodies against cytokeratins, has proven to be feasible and of prognostic value in breast, gastric, colorectal and lung cancer (30-33, 35-40). Immunocytochemistry is considered to be less sensitive, but more specific than molecular PCR detection (51) (although also a detection level of one tumor cell in $1x10^6$ normal cells has been described (52). Certainly, the morphological tumor cell assessment increases the specificity of this method. Sensitivity is perhaps hampered by the sample size to analyze. However, molecular detection of cellular expression of epithelial markers has recently been shown to harbor its own risks (53), particularly regarding specificity. Fifty-three control bone marrow samples were compared to 63 samples of patients with breast or prostate cancer. Commonly used markers as CK 18, CEA and PSA were evaluated. Only PSA mRNA was not detected in any of the control samples, but the other markers were (CK18: 5 out of 7 control samples positive). It was stated that limiting factors in the detection of micrometastatic tumor cells by RT-PCR are: the illegitimate transcription of epithelial genes in haematological cells and the varying

expression of the marker gene in micrometastatic tumor cells. Also (low level) expression of cytokeratin 19 (commonly used in the detection of micrometastatic breast cancer), was described to be detected in control tissues (54, 55). Possibly, low background expression of these markers in bone marrow, PBSC or peripheral blood can presumably be circumvented by quantifying the signal of the analyzed sample. In our center, a quantitative RT-PCR assay was developed for the gene encoding for the epithelial related membrane antigen EGP-2 (56). EGP-2 is one of the most tissue specific epithelial markers known so far, and is widely expressed on almost all carcinomas derived from simple epithelia (57). The EGP-2 molecule is not shed into the circulation. There is a monoclonal antibody available against the antigen for which this gene is encoding (58). With the quantitative RT-PCR for EGP-2, a base-line for background signals can be established, allowing a more meaningful interpretation of RT-PCR results.

Considering the technical challenges of detecting vary rare tumor cells in peripheral blood, it is not surprising that the data on this subject are limited. In one study, the specificity issue of RT-PCR appeared to have been overcome (59). With the detection of the maspin-transcript, tumor cell contamination was found in peripheral blood of 3 out of 9 stage IV breast cancer patients, particularly during systemic treatment. However, the role of this unusual marker remains unclear; no other research groups have validated this method so far. Recent data in breast cancer patients have all employed the classical CK19 marker(60-63): some have used an RT-PCR method (60), while most have combined PCR methods with immunostaining (61-63). In patients without distant metastases, an incidence of patients with positive peripheral blood samples of 5 to 9% with immunostaining, and 13 to 36% with (q)RT-PCR was reported (60-62). In one study, RT-PCR positive blood samples was associated

with distant metastatic versus node-negative or node-positive disease (60), but these results were not quantified or related to immunostaining. In metastatic breast cancer patients, the reported incidence of blood samples positive for CK19 mRNA expression by qRT-PCR was up to 50%, and decreased with disease response (63).

Concluding, it can be said that evaluation of the possibilities to measure micrometastatic disease in peripheral blood is ongoing. The low incidence of these cells and technical issues render this issue particularly challenging. When the prognostic value of peripheral blood contamination is further evaluated, this could be a helpful tool in the allocation of therapy to poor prognostic groups. Possibly, also a disease stage amendable for immunotherapy can be distinguished. The use of cellular and molecular detection may thus be used in allocating and evaluating new clinical approaches.

4.4 Peripheral blood stem cells

Tumor cell contamination of peripheral stem cell harvest has gained considerable interest, as the number of cancer patients treated with high-dose chemotherapy and stem cell support has increased steadily over the past decade. In breast cancer patients, Ross et al. reported on a study in which paired samples of bone marrow and peripheral stem cell harvest from 48 patients were analyzed with an immunocytochemical technique (25). In cell seeding experiments with a cocktail of monoclonal antibodies, one tumor cell per $5x10^5$ mononuclear cells was detected in bone marrow or peripheral stem cell harvest. Immunostained tumor cells were detected in 9.8% (13/133) peripheral stem cell specimens from 9/48 patients obtained after chemotherapy and a haematopoietic growth factor and in 62.3% (38/61) bone marrow specimens from 32/48 (66.7%) patients. It was concluded that peripheral stem

cells contain fewer tumor cells than paired bone marrow specimens from patients with advanced disease and that these cells appear, based on the clonogenic assay, to be capable of clonogenic tumor growth. Brügger et al. described a study in which a small number of patients was analyzed. In their immunocytochemical assay they also use a panel of monoclonal antibodies with detection of one tumor cell per $4x10^5$ normal cells. They found that there was a difference in appearance of the tumor cells in the circulation after chemotherapy and haematopoietic growth factor treatment between patients without bone marrow infiltration and with bone marrow involvement. In those without tumor involvement in the bone marrow, tumor cells appeared earlier in the circulation after chemotherapy and growth factor than in those with tumor contamination of the bone marrow (26). From this study it was clear that chemotherapy and growth factors resulted in a higher frequency of tumor cells in the circulation than without these compounds. The appearance of tumor cells in the circulation in patients with bone marrow contamination with tumor coincided with the appearance of peripheral stem cells. It was suggested that, possibly, tumor cells that metastasize to bone marrow may share some of the characteristics of haematopoietic progenitor cells, such as homing receptors/adhesion molecules. It may well be that there is a downregulation of adhesion molecules of stem cells by chemotherapy and growth factors, similar as occurs in normal maturation, but now happening at an immature stage. The fact that tumor cells can appear in the circulation without tumor cell contamination in the bone marrow suggests that either the assay to detect tumor contamination of the bone marrow was insensitive or that chemotherapy plus haematopoietic growth factors also mobilize tumor cells from other spots in the body. This raised the question whether this would also be the case in tumor types that do in general not metastasize early to the bone marrow, but in which high-dose

chemotherapy is considered to be potentially useful, such as in ovarian carcinoma. In a study addressing this issue, bone marrow and PBSC samples from 22 ovarian cancer patients were analyzed. No tumor cells were found in PBSC, but 47% of bone marrow samples stained positive. The exact influence of mobilizing regimes on stem cell contamination in this setting, remains to be established (64). In breast cancer, the influence of mobilizing stem cells with growth factor was compared to growth factor combined with chemotherapy (65). Immunocytochemical detection and clonogenic assays were used. Of stage IIIb or IV breast cancer patients receiving only G-CSF, 1 of 37 peripheral blood samples, 4 of 36 bone marrow samples and 2 of 38 PBSC samples were positive. Results were similar in the group receiving GM-CSF and cyclophosphamide, implying no additional role for chemotherapy in clearing tumor cells. Recently however, a number of reports contradicting this finding were presented. In 329 breast cancer patients, mobilization of peripheral blood stem cells with cytokine plus chemotherapy resulted in less tumor cell contamination (11.7%), than mobilization with cytokines alone (27.8%) (66). Also, a significant reduction of tumor cell contamination was seen when harvesting stem cells after the third course of chemotherapy for stage IV breast cancer was compared to harvesting after the first ($p=0.0052$) (67). The prognostic value of immunocytochemical detection of tumor cells in bone marrow and PBSC was evaluated in a fairly large number of stage IV breast cancer patients (68). A cocktail of antiepithelial antibodies was used. Patients without bone marrow and PBSC contamination had a significantly longer disease free survival than others with positive bone marrow and/or PBSC (471 days vs 339 days). It was concluded that immunocytochemical staining is a useful prognostic marker for autologous stem cell transplant. Although the significance and prognostic value of the detection of tumor cells in PBSC still remains to be clarified (69), it seems conceivable

that reinfusing tumor cells into the patient will affect the clinical outcome. Indeed, two studies (70, 71), strongly support the assumption that these malignant cells reinfused after high-dose chemotherapy might contribute to relapse. In both studies, grafts were marked by retroviral vectors encoding neomycin phosphotransferase or other foreign genes. These marker genes could be detected in the malignant cells in a majority of patients at relapse. These data have reinforced the need for efficient techniques for purging tumor cells from stem cell material, to reduce the risk of relapse after transplantation.

4.5 Purging

Measures to eliminate malignant cells from the graft are generally referred to as 'purging' (72). Ex vivo elimination of tumor cells is reasonable if there are no adverse effects on engraftment, haematopoietic and immune reconstitution, or other treatment outcomes. Evaluation of the efficacy of purging is difficult, as relapse may originate from residual disease in the patient as well as from malignant cells reinfused with the transplant. It is not known yet to what extent tumor cells should be depleted from the autograft, which is presumably strongly depending on the clonogenic ability of these single tumor cells. Basically, two methods for purging have been studied: depletion of tumor cells and selection of stem cells from the graft (73). Depletion was first studied using chemotherapy, mainly 4-hydroperoxycyclophosphamide (4-HC) (74, 75). Treatment with 4-HC clearly reduced in vitro tumor colony growth, but also colony formation and engraftment (76). In searching for more specific purging methods, immunotherapy using monoclonal antibodies were studied. For this approach to be effective, the antibody should be specifically reactive with tumor cells. Similar as with immunocytochemical staining, this is a difficulty in solid tumors. After reacting with

the antibody, additional steps are necessary to eliminate the tumor cells from the graft, either through cytotoxicity, immunotoxicity or immuno (magnetic) separation. When lymphoma cells were purged from bone marrow through an antibody-complement combination (41), a 3 to 6-log destruction was obtained. Disease free survival was increased in patients who received purged bone marrow, compared to those who did not. In a study by Mykleburst et al., the efficiency of immunotoxins and immunobeads for purging breast cancer cells from bone marrow were compared (77). The use of three monoclonal antibodies and immunomagnetic beads removed up to 6-log units of tumor cells. Immunotoxin efficacy was more variable, but both methods only slightly affected colony formation in bone marrow. Especially, a combination of antibodies on precoated immunobeads and two treatment cycles appears effective (72), but in these experimental settings still very high effector:target ratios are being employed. Also, these studies are performed with cell lines which are immunophenotypically well characterized. The efficacy in clinical settings with presumably a less homogeneic tumor cell population, remains to be established. Furthermore, an immunoselection method that eliminates 100% of tumor cells predictably, has not yet been described. To increase cytotoxicity, the use of cytokines has been studied. Especially the use of interleukin-2 (IL-2) in this setting seems promising. Whether IL-2 incubation of PBSC could induce tumor cell kill was studied by Verma et al. (78). Cytotoxicity was obtained with up to 50% tumor cell kill. No adverse effect was seen on colony formation of the PBSC. If this effect can be obtained with IL-2 alone, it is tempting to speculate on an increased effect with additional monoclonal antibodies. Future possibilities also include stimulation of the graft with GM-CSF, to induce monocyte-mediated cytotoxicity, and enhance cellular cytotoxicity (79). Apart from increasing purging efficacy in vitro, these approaches may also contribute to a possible graft versus host

effect. Residual tumor cells in the patient may thus be attacked. Post-autologous bone marrow transplantation administration of GM-CSF in vivo was shown to result in increased cytotoxicity, evaluated in vitro (80). In a similar setting, post-transplant administration of IL-2 is currently being evaluated (81). It will be very interesting to see whether from future studies an optimal time schedule (during mobilization in vivo, culturing of the graft in vitro, post transplantation in vivo) and an optimal combination of cytokines and perhaps antibodies can be determined, to increase cytotoxic efficiency in this particularly interesting clinical setting. The second approach for obtaining purified stem cells is to actually select out stem cells. Systems for selecting CD34 expressing haematopoietic progenitor cells, have been evaluated and are now available for large scale purification (82, 83). Engraftment of progenitor cells is not affected by this selection procedure (83, 84). Although the CD34 antigen is not detected on tumor cells from patients with most solid tumors (82, 83), a recent report indicated that tumor cells were still detectable after CD34 enrichment (85). Therefore, it is conceivable that a combination of purging strategies including a positive selection (of CD34 positive cells) and a negative selection (of tumor cells) would be the best approach to eliminate tumor cells from the transplant. This approach however, is time- and resources consuming, and through randomized trials more insight should be gained as to the actual benefits. To date, no randomized trials have addressed the long-term effects of CD34 selection in the adjuvant breast cancer setting. In metastatic breast cancer, the available data do either not allow conclusions on long-term impact of CD34 selection (because of too short follow-up, in a randomized trial, 86), or they suggest no beneficial effect (87). However, it should be reminded that a fundamentally different residual disease status between transplantation in the metastatic setting or in the adjuvant setting exists, and that therefore the metastatic setting may not have

predictive value for adjuvant treatment. One other important point about CD34 selection is that natural killer cells and T cells will be absent from the graft (73). Whether or not this will affect clinical outcome as a result of regrowth of residual cancer cells is not known. However, it is clear that immunological methods to increase cytotoxic effects as a graft versus host (e.g. residual disease) may be greatly hampered by this depletion. The impact of residual disease in the patient after high-dose chemotherapy and stem cell support, is as yet unknown (88). If the patient is the main source of residual tumor cells that cause relapse, this may be a rationale for adjuvant treatment with immunotherapy. The stage of minimal residual disease seems well suited for this treatment modality, as was shown by Riethmüller et al., for colorectal patients (89). How, in the setting of stem cell transplantation this can be combined with pretreatment of the graft to increase immunological efficiency, is of particular interest, but remains to be clarified.

4.6 Conclusion

Tumor cell detection is of particular interest in the setting of high-dose chemotherapy and peripheral blood stem cell support. It is well conceivable that reinfused tumor cells of the graft contribute to relapse, but the impact of residual disease in the patient in this setting remains to be established. A number of strategies are currently employed to purge tumor cells from stem cells. Presumably, a combination of these will be most effective in eliminating tumor cells. Short term effects of these purging procedures regarding engraftment, appear not harmful. Whether long term adverse effects will also be negligible, remains to be clarified.

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Epithelial glycoprotein-2 as a marker for minimal residual disease in breast cancer patients

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Abstract

Introduction Detection of single tumor cells may be helpful in breast cancer (BC) treatment. In this study, a quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) for the expression of membrane marker epithelial glycoprotein-2 (EGP-2) was evaluated for detecting BC cells in blood. **Materials and methods**: Sensitivity and specificity of the qRT-PCR was established in control nucleated blood cells, with or without EGP-2 positive MCF-7 tumor cells. The qRT-PCR was performed on breast tumors to determine a 'cut-off point' for EGP-2 expression in blood samples. Samples were also immunocyto(-histo)chemically (IC) stained with antibodies against EGP-2 (MOC31) and cytokeratin (CK) 19. **Results**: qRT-PCR sensitivity was 5-10 MCF-7 in 1.10^5 nucleated cells. Control samples without MCF-7 showed no (n=7) or low EGP-2 expression (n=3). EGP-2 expression varied 100-fold in breast tumors (n=12). IC sensitivity was 1 MCF-7 in 2.10^6 nucleated cells, and control samples were negative with MOC31. Aspecific staining was found with CK19. **Conclusions**: EGP-2 IC is more specific and sensitive than EGP-2 qRT-PCR or CK19 IC in this study. PCR methods for detecting BC cells in blood may be hampered by varying expression of tissue specific markers in BC tumors.

Introduction

Breast cancer patients without apparent distant metastases, who are treated with a curative intent, may later develop a relapse. Subgroups benefit from adjuvant chemotherapy treatment. The ability to detect very small numbers of tumor cells may provide the clinician with an important predictive tool with respect to recurrence and might help in a better selection for adjuvant therapy (33, 5). The detection of micrometastases in bone marrow was described to be of prognostic value in a number of solid tumors such as breast, gastric, colorectal and lung cancer (1, 3, 4, 7, 11, 23, 24, 29-31, 34). A particular challenge in solid tumors is to find a specific detection marker which is not shed into the circulation, which is not expressed by hematological cells and can therefore be used for tumor cell detection in blood, bone marrow or peripheral blood stem cells. For breast cancer, so far no tumor specific marker has been described. Many studies have been performed using tissue specific, epithelial markers (1, 3, 7, 11, 31). Sensitive detection methods such as RT-PCR, were found to detect low levels of background (or 'illegitimate') expression in non-epithelial tissue, including bone marrow and peripheral blood stem cells (21, 37) with these markers. To circumvent this problem, a quantitative RT-PCR (qRT-PCR) was developed in our institution for the expression of epithelial glycoprotein-2, EGP-2 (15). EGP-2, also called Ep-CAM, is a 40-kDa membrane-bound glycoprotein, strongly expressed by most carcinomas and universally expressed in breast cancer specimens (18). As such, EGP-2 is a commonly used target antigen in many carcinoma-directed immunotherapeutical approaches (10, 18, 32). In this study, this qRT-PCR method was evaluated for detecting of breast cancer cells in blood. To this end, the sensitivity and specificity were studied, as well as the EGP-2 expression in breast samples. Results were compared to immunocytochemistry (IC) by means of antibodies against EGP-2 (MOC31) and cytokeratin (CK) 19, a tissue specific marker commonly used for this purpose (20, 22, 27).

Materials and methods

Isolation of nucleated cells from blood samples

Blood samples (of 40 mL each) were collected in tubes containing ethylenediaminetetraacetate (EDTA) as anti-coagulans, after prior collection of a waste amount of sample (2 mL). Samples were put on ice immediately. Erylysis was performed with an ammonium chloride solution (155 mM NH_4Cl, 10 mM potassium hydrogen carbonate, 0.1 mM sodium ethylene diamine tetra acetate, EDTA). The cells to be used for RNA analysis (3/4 of the total amount of cells) were transferred into a guanidinium-isothiocyanate solution (GITC solution: 4 M guanidinium-isothiocyanate, 0.5% n-lauroyl sarcosine, 25 mM sodium-citrate pH 7.0 and 0.1M β-mercaptoethanol) and kept at -20°C, until further processing.

The cells to be used for IC (1/4 of the total amount of cells) were allowed to sediment for

1 h onto slides, through a metal funnel-like device. This sedimentation method was found to introduce less mechanical damage for the cells than the standard cytospin method. Also, it allows the analysis of 1.10^6 cells on one slide (instead of 5.10^4 on a cytospin slide). Fixation of the cells was then performed for 10 min at -20°C, in a solution of 4% acetic acid in methanol, followed by 10 min at -20°C, in acetone. Because standard acetone fixated cytospins were found to result in poor leukocyte morphology, and the morphological distinction between normal cells and tumor cells was thought to be important in this type of study, the above described alternative sedimentation- and fixation procedure was developed. Slides were maintained at -20°C until further processing.

Nucleated cells from blood samples were used from healthy volunteers for spiking experiments with breast cancer cell line MCF-7 in order to determine the sensitivity of the qRT-PCR and IC methods.

The specificity of the methods was evaluated in samples from healthy volunteers used as a negative control.

Primary breast tumor samples

As a part of this study, fresh tumor samples from primary surgery in breast cancer patients were snap frozen and maintained at -80°C. Prior to RNA isolation or IC staining, 25 µ slices were prepared. Tumor content was verified with hematoxylin-eosin (HE) staining of slices on either side of the sample, and examination of these slides by a technician as well as a pathologist. Slices for RNA isolation were transferred immediately into GITC and homogenized through a 20-Gauge syringe; slices for staining were transferred onto slides. Benign breast samples were obtained from patients undergoing prophylactic mastectomy procedures, and were processed in the same way as the tumor samples. The collection of patient samples from breast cancer patients for studying circulating tumor cells was approved by the Medical Ethical Committee or our institution.

The expression of EGP-2 in primary breast samples was analyzed by means of the qRT-PCR, with the aim to use this information for establishing a cut-off point for the expression of EGP-2 in blood samples.

RNA isolation

RNA isolation was performed by means of chloroform/isopropanol extraction (6). Samples were maintained in 500 µL GITC, at -20°C. After thawing, 50 µL sodium acetate (3M,pH 5.0), 500 µL water saturated phenol and 100 µL chloroform/isoamyl alcohol (49:1 vol:vol) were added and mixed by vortexing. The mixture was left on ice

for 10 min, after which it was centrifuged, 15,000 g, at 4°C. The supernatant was transferred into a fresh microfuge tube, and RNA was induced to precipitate by adding an equal volume of isopropanol and placing it at -20°C for 1 h. RNA was pelleted by centrifugation, 15,000 g, at 4°C, and re-precipitated in 150 µL GITC and isopropanol. RNA was pelleted by centrifugation as described above, and washed with 70% ethanol. The pellet was dried in a vacuum exicator and dissolved in 30 µL diethylpyrocarbonate (DEPC, Sigma, St. Louis, MO) treated water. The integrity of the RNA samples was assessed on formaldehyde-containing (2.2 M) agarose gels. RNA samples with visible and discrete 28S and 18S ribosomal RNA bands were used for the RT-PCR experiments.

Quantitative RT-PCR on EGP-2:

The qRT-PCR on EGP-2 was performed according to Helfrich et. al (15). The EGP-2 specific primers were synthesized on an oligonucleotide synthesizer (Pharmacia Biotech Europe, Brussels, Belgium), using the phosphite triester method. The sequences of the EGP-2 specific primers are: EGP2 FW: 5'-GAACAATGATGGGCTTTATG-3' (corresponding to bases 374 to 394 of the EGP-2 cDNA) and EGP2 REV: 5'-TGAGAATTCAGGTG-CTTTTT-3' (bases 868 to 888). Amplification of cDNA with these primers gives rise to a 513 bp fragment. No signal can be obtained with genomic EGP-2 with these primers. For quantifying the EGP-2 signal, a deletion construct of recombinant EGP-2 RNA was used. This construct yields a 315 bp fragment when subjected to PCR amplification with EGP-2 specific primers, which can be readily distinguished from the 513 bp fragment generated by genuine EGP-2 cDNA on an 1.5 % agarose gel. A series of recRNA solutions, decreasing in concentration, was mixed with a fixed amount of cellular RNA, containing the EGP-2 RNA to be quantified. The mixture of recRNA and cellular RNA was converted into cDNA by reverse transcription using the EGP-2 REV primer. cDNA was then subjected to 30 PCR cycles consisting of 30 sec denaturation at 94 °C, 60 sec

of primer annealing at 54 °C, and elongation at 72 °C for 90 sec. The samples were subjected to an initial denaturation for 180 sec. The final elongation step was extended by 10 min. Reactions were performed using the DNA Thermal Cycler (Perkin Elmer Cetus, Norwalk, CT), with supertaq (0.125 U) (HT Biotechnology LTD), in the reaction buffer supplied by the manufacturer, supplemented with $MgCl_2$ (1.75 μM), 200 μM dNTP's, and 300 ng of both EGP2 FW and EGP2 REV. The MCF-7 breast cancer cell line was used as positive control. A sample to which all the above mentioned components were added, without RNA, was used as a negative control. The reaction products were analyzed by gel electrophoresis, and the weight ratios per lane were determined by densitometry. The lane in which the weight ratio was closest to the ratio of the respective RNAs was used to quantify the amount of EGP-2 mRNA. On all samples, RT-PCR was also performed to show glyceraldehyde-3-phophatase dehydrogenase (GAPDH) mRNA, with primers GAPDH FW: 5'-CCACCCATGGCAAATTCCATGGCA-3' and GAPDH REV: 5'-TCTAGACGGCAGGTCAGGTCCACC-3' (25 cycles). EGP-2 expression was normalized to expression of the house-keeping gene GAPDH, and expressed relative to GAPDH expression.

Immunocyto (-histo) chemical (IC) staining

Nucleated cell slides from blood samples were pre-treated with 0.3% hydrogen peroxide for 15 min to block endogenous peroxidase activity of nucleated cells, followed by rinsing with phosphate buffered saline (PBS) solution (0.14 M NaCl, 2.7 mM KCl, 6.4 mM $Na_2HPO_4.2H_2O$, 1.5 mM KH_2PO_4, pH 7.4). With each antibody, at least two of these slides (e.g. 2x 1.10^6 cells) were stained. Tumor samples in paraffin were pre-treated with protease (type XXIV, Sigma, St. Louis, MO), 0.1% in PBS pH 7.4, at 37°C, for 30 min, prior to staining. Samples were stained with monoclonal antibody MOC31, directed against EGP-2, using indirect immunoperoxidase staining with horseradish peroxidase conjugated rabbit anti mouse as a second antibody (Dako, Glostrup, Danmark) and AEC

as a substrate. Samples were routineously counterstained with HE. The antibody directed against cytokeratin was anti-CK19 (clone 170.2.14, Roche Diagnostics, Almere, The Netherlands), diluted 1:250 on slides and 1:500 on tumor samples in PBS 1% bovine serum albumin (BSA, Life Technologies, Breda, The Netherlands). Prior to staining with the CK19 antibody, slides were treated with a 0.01% trypsin (Life Technologies, Breda, The Netherlands) solution in 0.1% $CaCl_2$ in 0.1 M Tris (hydroxymethyl) aminomethane solution, pH 7.8, at 37°C for 5 min. Also for the CK19 antibody, the indirect immunoperoxidase staining procedure was used as described above.

Isotype specific controls for MOC31 and CK19 were performed with primary antibody mouse IgG1 (X0943, Dako, Glostrup, Danmark). In each staining procedure, a negative control was included using PBS 1% BSA without the primary antibody. Slides prepared from MCF-7 breast cancer cells (staining positively for CK19 and MOC31) were used as positive controls in each procedure for staining slides. For tumor samples, as positive control a sample of breast cancer tissue was used, that was found to stain positive with MOC31 and CK19 antibodies on previous occasions.

Patient samples were independently examined for morphological features by a technician as well as a pathologist.

The classical immunocyto (-histo) chemistry method was used as comparison with the (less established) qRT-PCR method.

Results

qRT-PCR for EGP-2 expression

Spiking experiments (sensitivity)

MCF-7 breast cancer cells were used for spiking in nucleated cell samples from healthy volunteers. Consistently, 5 to 10 MCF-7 cells in 1.10^5 nucleated cells could be detected by means of the qRT-PCR.

Healthy (negative) controls (specificity)

The specificity of the assays was examined using nucleated cells from healthy volunteers (n=10). The qRT-PCR showed no (n=7) or low expression (0-1 relative to GAPDH expression, n=3) of EGP-2. These results are in line with those described by de Graaf et al. (9).

Malignant and benign breast samples (cut-off point EGP-2 expression)

In primary tumor samples (n=12), a range of EGP-2 expression was found: mean 3.4, range 0.1- 11.5 relative to GAPDH expression. A representative picture of a gel reflecting a qRT-PCR of two tumor samples is shown in figure 1. Benign breast samples (n=3), all showed low EGP-2 expression of 0-1 relative to GAPDH expression.

IC with antibodies against EGP-2 (MOC31) and CK19

Spiking experiment (sensitivity)

In the same samples as used for qRT-PCR analysis, 1 MCF-7 tumor cell could be detected in the amount of nucleated cells screened, e.g. 2.10^6 total, with IC staining with the MOC31 antibody directed against EGP-2, as well as the anti-CK 19 antibody. In figure 2, an example is shown of MCF-7 tumor cells added to leukocytes, stained with MOC31.

Healthy (negative) controls (specificity)

IC with MOC31 staining was consistently negative in these samples (n=10). However, in some samples, slightly positive cells were detected with the anti-CK 19 antibody. These cells were judged to be segmented granulocytes.

Malignant and benign breast samples

Primary tumor samples (n=12), found morphologically malignant by regular HE staining, all stained positive with MOC31 and anti-CK 19 antibody. Epithelial tissue of benign breast samples (n=3) stained positive with the MOC31 antibody against EGP-2 and the anti-CK 19 antibody.

Discussion

In this study, we evaluated the use of a qRT-PCR for the expression of EGP-2 for detecting minimal amounts of breast cancer tumor cells in blood samples. The sensitivity as well as the specificity of the method were studied, and the expression of EGP-2 in breast samples was evaluated. Classical immunocyto (-histo) chemistry with antibodies against EGP-2 (MOC31) and CK19, was used as comparison with the molecular biological qRT-PCR method.

For the preparation of blood samples, the nucleated cell fraction was isolated by means of erythrocyte lysis. This method was recently shown in blood, bone marrow and leukapheresis products to preserve tumor cells in a superior way compared to the frequently used Ficoll isolation (19). To establish the sensitivity of the detection methods, spiking experiments with MCF-7 in nucleated cells from healthy volunteer blood samples were performed. With IC, a detection level with IC of 1 MCF-7 tumor cell in 2.10^6 nucleated cells was found, using the MOC31 antibody directed against EGP-2 or the anti-CK 19 antibody. The qRT-PCR resulted in a detection level of only 5 to 10 MCF-7 cells in 1.10^5 nucleated cells (in line with ref. 15): a 100-200 fold difference in sensitivity. In determining the specificity of the methods, the control samples of nucleated cells from healthy volunteers were found positive in 3 out of 10 cases, with a low EGP-2 expression. None of these samples stained positive with IC for MOC31.

It can be suggested, with the techniques used here, that this EGP-2 based qRT-PCR method may be less sensitive and specific than IC for MOC31, based on these results. Although early reports have suggested a high sensitivity (up to 1 tumor cell in 1.10^7 nucleated cells) of PCR based methods for detecting tissue specific expression of single solid tumor cells in blood of bone marrow samples (8, 12, 35), more recent reports indicate that these methods harbor the risk of false positive results (2, 9, 13,

16, 21, 25, 37). This may be due to detection of so called 'illegitimate transcription' of apparent tissue specific markers by non-epithelial cells, or due to the sensitivity of this method (particularly the nested RT-PCR) (36) to contamination. It is also clear that analytical variables of the RT-PCR methodology may have a profound impact on the obtained results (38). In an effort to circumvent the problem of false positive results with the regular RT-PCR, the qRT-PCR method for EGP-2 was designed in our institution (15). It was presumed that quantifying the signal of EGP-2 would allow the definition of a cut-off point from low-level or insignificant expression. With this method, it was found that within tumor cell lines, positive with IC for MOC31, a wide variation (of 100-fold) in EGP-2 expression could be detected (15). This is in line with EGP-2 expression results in tumor cell lines described earlier (9). If EGP-2 expression were to vary in primary tumor samples as well, establishing a cut-off point for EGP-2 expression, as well as relating EGP-2 expression to tumor load, would be difficult. A low expression of EGP-2 in blood or bone marrow might then reflect a relevant tumor load in one patient, but not in the other. Indeed, also in primary breast tumors we observed a 100-fold difference in expression of EGP-2, normalized for GAPDH expression. Thus, based on these results, we suggest that with this qRT-PCR for EGP-2, the definition of a generally applicable cut-off point for EGP-2 expression seems not feasible. Unless the expression of EGP-2 in primary tumor tissue and blood samples are related for each individual, it appears difficult to exclude false positive results with this method. Recently, the same problem of varying expression was described for primary colorectal tumors (26). Immunocytochemical detection methods, have the advantage of providing a visual evaluation of stained (tumor) cells, thus decreasing the risk of false positive results. In fact, it was recently suggested that recruitment of tumor cells into peripheral blood cannot be confirmed by RT-PCR alone, and that IC should be performed as validation (14). It can be argued that the IC method is considerably laborious and potentially sensitive to inter-observer variation in the

judgement of tumor cells. However, based on the results presented here, we currently prefer the use of the anti-EGP-2 antibody MOC31 for detection of single tumor cells in breast cancer patients over this qRT-PCR.

In light of the IC detection of single tumor cells, we also compared the EGP-2 to the commonly used marker CK19 (20, 22, 27). Aspecific staining of granulocytes was found in some samples from healthy volunteers. This is in line with a recent study performed by Lambrechts et al. (21), who found as much as 75% false positivity in breast cancer patient samples as well as samples from healthy volunteers, with the CK19 antibody. The fact that the cytokeratin epitope is found not only on all epithelia, but also on endothelia, mesothelia, skin and mucosa (17) may explain this higher rate of false positive results with CK19 than MOC31, as EGP-2 is only expressed on non-squamous epithelia (28).

Concluding, we suggest that the current classic molecular methods for detecting single breast cancer tumor cells are impeded by the lack of a tumor-specific marker for breast cancer cells. The use of fluorescent probes in quantitative RT-PCR may enhance tumor cell detection to the level of IC; this method is possibly the least laborious method available. Currently, research is in progress to evaluate this issue. For now, IC based on tissue-specific markers, has the advantage of allowing an additional visual evaluation of the stained (tumor) cells. When using IC staining, the EGP-2 marker yields more specific results than CK19.

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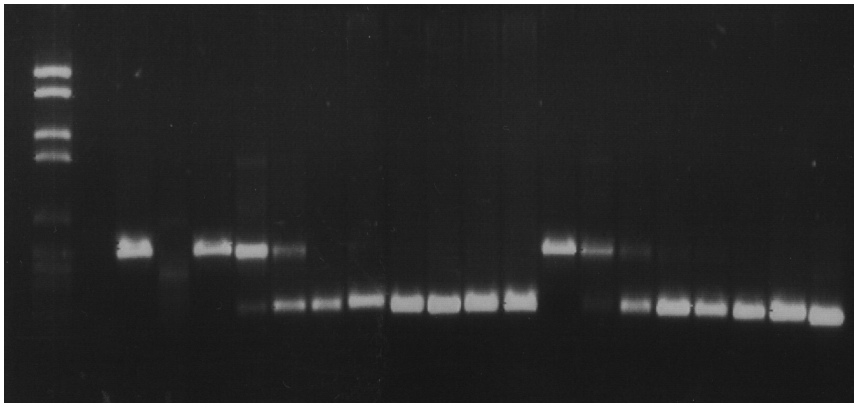

Figure 1:

A representative gel reflecting the qRT-PCR of two malignant breast tumors. M: marker; 1: negative control without RNA; 2: positive control of MCF-7 tumor cells; 3: negative control of GLC4 tumor cells; lanes 4-12 and lanes 13-20: two separate tumor samples to which dilutions of recombinant RNA was added (ranging from 0 to 1000 pg: lanes 4 as well as 13, and lanes 12 as well as 20 respectively).

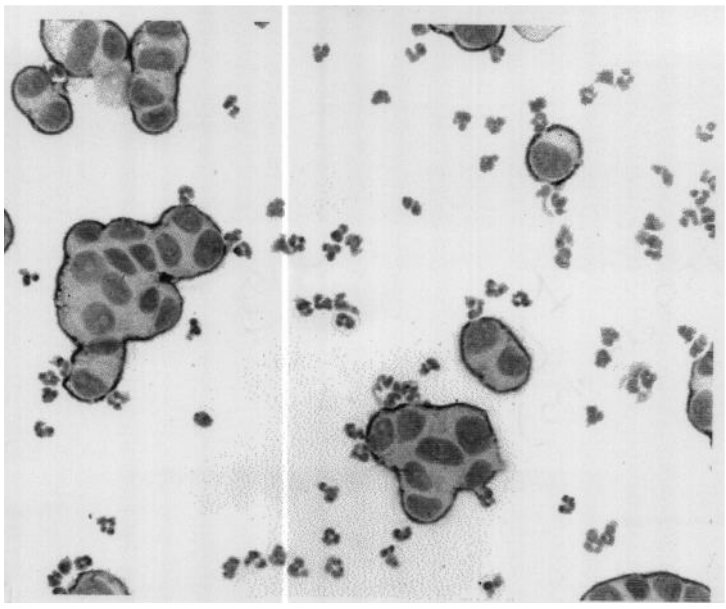

Figure 2:

Representative picture of MCF-7 tumor cells added to leukocytes for spiking purposes, stained with MOC31 antibody. Magnification 40x10.

Detection of micrometastatic breast cancer by means of real time quantitative RT-PCR and immunostaining in perioperative blood samples and sentinel nodes

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ABSTRACT

Introduction: the predictive value of conventional staging in breast cancer is limited, and more sensitive staging methods may be valuable in selecting patients for adjuvant systemic therapy. The aim of this study was to detect cells positive for epithelial glycoprotein-2 (EGP-2) and cytokeratin 19 (CK19), using immunostaining and real time quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR). **Patients and methods**: from 58 breast cancer patients, 52 primary tumors, 75 sentinel nodes (SN) and 149 peripheral blood (PB) samples (from before, during and 4 days after operation) were examined. Immunostaining was performed with antibodies directed against EGP-2 and CK19. Detection limits were 1 MCF-7 breast cancer cell line cell/2.10^6 leukocytes (immunostaining) and 1 MCF-7 cell/10^6 leukocytes (qRT-PCR). Control non-cancer lymph nodes (10) showed aspecific CK19 staining, but were qRT-PCR negative; control healthy volunteer PB (11) was always negative. **Results**: primary tumor samples, all positive with immunostaining, showed a wide variation of EGP-2 (>10^4 fold) and CK19 mRNA expression (>10^3 fold). SN (n=19) from 16 patients were tumor-positive with routine haematoxylin-eosin (H&E) and/or immunostaining. SN tumor presence was positively correlated to qRT-PCR expression, but 3 tumor-positive SN were false negative with qRT-PCR. Three SN were qRT-PCR positive, while tumor negative with H&E and/or immunostaining. No immunostaining positive PB was observed, but 19 patients (33%) had one or more qRT-PCR positive PB samples. **Conclusions**: Primary tumors have varying expressions of EGP-2 and CK19 mRNA. Both markers can be used in qRT-PCR to obtain adequate sensitivity for single tumor cell detection. In SN, immunostaining appears more sensitive/specific than H&E or qRT-PCR for tumor detection. In PB, no immunostaining positivity was found, while 33% of patients had qRT-PCR positive PB. The clinical implications of these findings will have to be clarified in large studies with long-term clinical follow-up data.

INTRODUCTION

Staging of breast cancer patients, to determine prognosis and treatment, is largely based on assessment of tumor size and axillary node-status. However, the prognostic value of finding node-negative cancer based on conventional analysis is limited, and distant metastases develop in 20 to 30% of women with negative nodes (1). Although primary tumor characteristics may be helpful in selecting patients at risk of metastatic disease (2), the identification of new markers which predict the necessity of giving early adjuvant systemic treatment has gained much interest in the last decade. In view of this, the ability to detect micrometastases in lymph nodes, bone marrow and blood was examined in recent years (3-5). Careful evaluation of tumor cell presence in the sentinel lymph node, the first node in the lymphatic basin of the breast tumor, is particularly important. Removal of the sentinel node is increasingly used as an alternative staging procedure for total axillary lymph node dissection (6), in view of decreased morbidity in breast cancer patients. In addition to evaluation of the presence of lymph node micrometastases, it may be useful to examine early hematogenous spread of tumor cells. Recent immunocytochemical studies (7, 8) indicated that the ability of breast tumor cells to expand to the bone marrow hematogenously is independent of their ability to metastasize to axillary lymph nodes. Detection of micrometastastic disease by means of immunostaining or PCR based methods have been examined. The limitations of these methods have become clear over the last years. Conventional immunostaining techniques allow assessment of the actual nature of stained (tumor) cells, but appear to be very laborious (3-5). RT-PCR based methods, targeting tumor-associated, tissue specific antigens, were first considered to have a high sensitivity (9-11), but were found later to harbor the risk of giving false positive results (12-21).

The aim of the present study was to evaluate the use of two markers: epithelial glycoprotein-2 (EGP-2) as well as cytokeratin 19 (CK19) using immunostaining as well as a real time quantitative RT-PCR, and to relate this to standard morphology assessment, in the detection of micrometastatic breast cancer. EGP-2, also called Ep-CAM, is a 40-kDa membrane-bound glycoprotein. As a pan-epithelial/carcinoma marker, EGP-2 is universally expressed in breast cancer specimens (22). It has been used as a target antigen in a number carcinoma-directed immunotherapeutical approaches (23-24), as well as for detection of single tumor cells (25). CK19 is an intracellularly located tissue-specific marker, commonly used for detection of micrometastatic disease in solid tumors (3-5). The analyses were performed on a unique series of corresponding primary breast tumors, sentinel nodes and sequential (perioperatively collected) peripheral blood samples. The parallel use of immunostaining as well as a qRT-PCR with two well known markers for cancer detection, on a large collection of patient samples has not been described before. This setting provides an opportunity to study the value of these methods in detecting micrometastases in breast cancer patients.

PATIENTS AND METHODS

Patients

Between April 1997 until July 1999, patients undergoing primary breast tumor surgery and sentinel lymphadenectomy in the University Hospital Groningen were eligible for the present study, which was approved by the Medical Ethical Committee of our institution. Written informed consent for the collection of samples was obtained from all participating patients. The sentinel lymphadenectomy procedure (26, 27) is performed since October 1996 at the University Hospital Groningen, on patients with an operable breast tumor that appeared malignant on clinical examination, imaging (mammography, ultrasonography or both) and fine-needle aspiration cytology, without clinically suspect axillary lymph nodes (28). Staging of patients was performed according to the TNM system (Union Internationale Contre le Cancer, 1997).

Primary tumor samples

Fresh tumor samples were placed on ice directly following surgery. One part of the tumor was subsequently snap frozen, and the other part was formalin fixed and paraffin embedded for standard H&E staining and histological examination, including determination of grading. The snap frozen sections were maintained at -80°C until further use. Prior to RNA isolation or immunostaining, 25 µm sections were prepared. The presence of tumor was verified using H&E stained sections on either side of the sample. Sections for RNA isolation (ten 25 µm sections per tumor sample of approximately the same size) were transferred immediately into a guanidinium-isothiocyanate solution (GITC solution: 4 M guanidinium-isothiocyanate, 0.5% n-lauroyl sarcosine, 25 mM sodium-citrate pH 7.0 and 0.1M β-mercaptoethanol) and homogenized through a 20-Gauge syringe. Sections elected for immunostaining were transferred onto slides.

Sentinel nodes

The sentinel node was removed after localization of the node through intralesional injection of patent blue dye (Bleu Patenté V, Laboratoire Guerbet, Aulanay-sous-Bois, France) and/or radioactive tracer (Nanocoll; Sorin Biomedica Diagnostics, Sallugia, Italy), and subsequent detection using the hand-held γ-detection probe (Neoprobe, Neoprobe Corporation, Dublin, OH) (26). Fresh sentinel nodes were placed on ice directly following surgery. Each node was divided in two pieces. One piece was paraffin embedded for routine pathological examination, including step sections and immunostaining using the pan-keratin antibody CAM5.2 (Becton Dickinson Benelux, Bilthoven, the Netherlands). The other piece was snap frozen. Sentinel nodes were further prepared and handled as described for the primary tumor. Control non-cancer lymph nodes (n=10) were processed and analyzed identically to the sentinel nodes.

Collection and isolation of nucleated cells from blood samples

Blood samples were collected prior to surgery (t_0), following tumor removal during surgery (t_1) and four days after surgery (t_2). Samples (40 mL) were collected in tubes containing ethylene diamine tetra acetate (EDTA) as anti-coagulant, after prior collection of a waste amount of sample (2 mL). Samples were put on ice immediately. Erylysis was performed with an ammonium chloride solution (155 mM NH_4Cl, 10 mM potassium hydrogen carbonate, 0.1 mM sodium EDTA). This method was shown to preserve possible tumor cells in a superior fashion compared to the frequently used Ficoll method (29). The cells to be used for RNA analysis (3/4 of the total amount of cells) were transferred into GITC, and kept at -20°C, until further processing. Control blood samples were collected from healthy volunteers, and processed in the same fashion as described above, to be used as negative controls in the qRT-PCR method described below. Also, the sensitivity of this qRT-PCR method was determined in control

healthy donor leukocytes in which EGP-2 and CK19 positive breast cancer cell line MCF-7 cells were added in various dilutions.

The cells to be used for immunostaining (1/4 of the total amount of cells) were allowed to sediment for 1 hour onto slides, through a metal funnel-like device, allowing the analysis of 1.10^6 cells on one slide. Cells were fixed at -20°C for 10 minutes, in a solution of 4% acetic acid in methanol, followed by 10 minutes at -20°C, in acetone. Slides were stored at -20°C until further processing. Of each patient sample, one slide was used for Giemsa staining to assess morphology.

Immunostaining

Nucleated cell slides from blood samples were pre-treated with 0.3% hydrogen peroxide for 15 minutes to block endogenous peroxidase activity of nucleated cells, followed by rinsing with phosphate buffered saline solution (PBS, 0.14 M NaCl, 2.7 mM KCl, 6.4 mM $Na_2HPO_4.2H_2O$, 1.5 mM KH_2PO_4, pH 7.4). At least two of these slides (e.g. 2x 1.10^6 cells) were immunostained with each primary antibody. Samples were stained with monoclonal antibody directed against EGP-2 (MOC31), using an indirect immunoperoxidase staining with horseradish peroxidase conjugated rabbit anti mouse as secondary antibody (Dako, Glostrup, Denmark) and 3-amino-9-ethylcarbazole as substrate. Samples were routineously counterstained with haematoxylin. The antibody directed against cytokeratin19 was anti-CK19 (clone 170.2.14, Roche Diagnostics, Almere, The Netherlands), diluted 1:250 on slides and 1:500 on tumor samples in PBS, 1% bovine serum albumin (BSA, Life Technologies, Breda, The Netherlands). Prior to staining with the CK19 antibody, slides were pre-treated with a 0.01% trypsin (Life Technologies, Breda, The Netherlands) solution in 0.1% $CaCl_2$ in 0.1 M Tris (hydroxymethyl) aminomethane solution, pH 7.8, at 37°C for 5 minutes, and the indirect immunoperoxidase staining procedure was used as described above.

Isotype specific controls for MOC31 and CK19 were performed using primary antibody mouse IgG1 (X0943, Dako, Glostrup, Denmark). In each staining procedure, a negative control was included using PBS, 1% BSA without the primary antibody, whereas slides prepared to contain MCF-7 cells were used as positive controls. For tumor samples, as positive control in each procedure, a sample of MOC31 and CK19 positive breast cancer tissue was used. Patient samples were examined independently for morphological malignant features by a technician as well as a pathologist.

In slides containing leukocytes from 11 healthy controls, no CK19 or MOC31 positive cells were observed. In spiking experiments of MCF-7 cells, diluted in leukocytes, 1 tumor cell could be detected in a total of 2.10^6 leukocytes (e.g. the total number of cells analyzed).

RNA isolation

RNA isolation from all samples was performed by means of the Rneasy Mini Kit (Qiagen, Westburg b.v., Leusden, The Netherlands), according to the manufacturer's instructions. Briefly, 350 µL of cell lysate (or a quantity corresponding with 5.10^6 nucleated cells, in the case of the blood samples) was obtained. One volume of 70% ethanol was added to the lysate, and mixed well by pipetting. The mixture was applied to a spin column, to allow adsorption of RNA to the membrane, and the column was subsequently centrifuged at 8,000 g, for 30 seconds. Columns were washed and the RNA sample was eluted from the column by means of RNase free water, after centrifugation at 8,000 g for 1 minutes, and maintained in 50 µL Rnase free water.

The integrity of the RNA samples was assessed on formaldehyde-containing (2.2 M) agarose gels. Only RNA samples with visible and discrete 28S and 18S ribosomal RNA bands were used for the RT-PCR experiments.

Real time quantitative RT-PCR (qRT-PCR) for assessment of EGP-2 and CK19 expression

The qRT-PCR was performed by means of the LightCycler (Roche Diagnostics, Mannheim, Germany), according to the manufacturer's instructions. The LightCycler system is based on the continuous monitoring of the formation of PCR product by measuring the amount of hybridization probes annealing to the target sequence in every cycle. Hybridization probes emit a fluorescent signal, that correlates to the concentration of target. The sequence of specific primers and probes was checked prior to use to avoid amplification of genomic DNA or pseudogenes (CK19), by means of software packages SeqMan 3.57 and PrimerSelect 3.10 (DNASTAR Ltd., London, U.K.). A volume of 5 µL total RNA in 20 µL master mix (Roche Diagnostics, Mannheim, Germany), 3 pmol of the specific primers and 2 pmol of the specific hybridization probes was added to each glass capillary. The sequences of the EGP-2 specific primers were: EX3F: 5'-GAACAATGATGGGCTTTATG-3' (corresponding to bases 374 to 394 of the EGP-2 cDNA) and EX7R: 5'-TGAGAATTCAGGTGCTTTTT-3' (bases 868 to 888). The sequences of the EGP-2 specific probes were: ALL-FL: ATCCAGTTGATAACGCGTTGTGAT-x and ALL-NEW: Red 640-TCCTTCTGAAGTGCAGTCCGCAA-p. The sequence of the CK19 specific primers were: CK19 F: 5'-ACTACAGCCACTACTACACGAC-3' (corresponding to bases 409-430), and CK19 R: CAGAGCCTGTTCCGTCTCAAAC (corresponding to bases 557-536). The sequences of the CK19 specific probes were: CK19-FL: TGTCCTGCAGATCGACAACGCCC-x (corresponding to bases 485-507), and CK19-705: Red 705-TCTGGCTGCAGATGACTTCCGAACCA-p (corresponding to bases 509-534). The detection of EGP-2 or CK19 target was performed in separate glass capillaries.

Samples were submitted to an initial reverse transcriptase hybridisation step of 25 minutes at 55°C, followed by a denaturation step of 30 seconds at 95°C, then amplification in a three-step cycle procedure (denaturation 95°C, ramp rate

20°C/second; annealing 56°C, 15 seconds, ramp rate 20°C/second; and extension 72°C, 20 seconds, ramp rate 2°C/second) for 45 cycles. Finally, a melting three step cycle was performed (95°C, ramp rate 20°C/second; 60°C, 10 seconds, ramp rate 20°C/second; 95°C, ramp rate 0.2°C). On several occasions, after the LightCycler procedure, the contents of the glass capillaries were also checked on gel, always conveying the expected PCR product for the specific primers in tumor-positive samples. In all runs, a standard curve was obtained using the same samples of RNA from dilutions of MCF-7 cells in healthy donor leukocytes (1:10; $1:10^2$; $1:10^3$; $1:10^4$; $1:10^5$; $1:10^6$ respectively), with a total of 1.10^6 cells. All results were always expressed in relation to the standard curve, e.g. the number of MCF-7 cells in a total number of 1.10^6 cells (leukocytes). Samples of primary tumor and sentinel nodes were checked for the presence of tumor, and corrections were made for the RNA expression of β-2 microglobulin. The sequence of the β-2 microglobulin specific primers were: β-2 microglobulin F: CCAGCAGAGAATGGAAAGTC (corresponding with bases 33 to 52) and β-2 microglobulin R: GATGCTGCTTACATGTCTCG (corresponding with bases 301-282). The sequences of the β-2 microglobulin specific probes were: β-2 microglobulin-FL: TTCTTCAGTAAGTCAACTTCAATGTCGGA-x (corresponding to bases 118-90) and β-2 microglobulin-705: LC Red 705-ATGAAACCCAGACACATAGCAATTCAG -p (corresponding to bases 86-60). As a positive control, the RNA from undiluted MCF-7 cells was used; as a negative control, a sample was used to which no RNA at all was added. Also in each experiment, a sample containing healthy donor leukocytes without tumor cells was included as a negative control.

With this method, a sensitivity was obtained of 1 spiked MCF-7 cell, detected in 1.10^6 healthy donor leukocytes (n=3 separate experiments). In leukocyte samples from n=11 healthy donors, the EGP-2 or CK19 signal remained below the detection limit.

Statistics

Statistical analyses were performed by means of the SPSS statistical software package. The relation of morphology H&E, immunostaining and qRT-PCR data (primary tumor, sentinel nodes and peripheral blood) and tumor size, grading and disease stage was analyzed by means of the Pearson correlation test. Comparisons of PCR expression data between the group with or without tumor (primary tumor, sentinel nodes and peripheral blood) were performed by the Mann-Whitney test. Only p-values <0.05 were considered significant.

RESULTS

Patients

From April 1997 until July 1999, 58 patients participated in this study. Patients' characteristics are given in table 1.

Primary tumors

From 57 patients, the primary tumor could be obtained; in one case, no primary tumor was removed. From 3 patients, 2 different tumor lesions were removed in the same surgical procedure (2 patients had a tumor in both left and right breast; 1 patient had two separate lesions in the same breast). From the total of 60 removed tumors (tumor characteristics shown in table 1), 52 contained sufficient material to allow snap-freezing, in addition to the material needed for establishing a pathological diagnosis.

Immunostaining: Tumor content of the frozen samples was verified using H&E sections; in 46 samples carcinoma was detected. In the other 6 samples, H&E examination showed in situ carcinoma in 2 lesions, whereas in 4 lesions no residual carcinoma could be detected. Immunostaining using the MOC31 or anti-CK19 antibody was positive in all frozen sections.

QRT-PCR: the mean mRNA expression of EGP-2 in malignant tumors was equivalent to the expression of 17,693 (SEM 4,995) MCF-7/10^6 leukocytes; the mean mRNA expression of CK19 was 12,382 (SEM 2,058) MCF-7/10^6 leukocytes. EGP-2 mRNA expression correlated with CK19 mRNA expression ($r=0.382$, $p=0.006$) (figure 1). In figure 1, also the wide range of both EGP-2 and CK19 mRNA expression in tumors is illustrated. The mRNA expression of either EGP-2 or CK19 was not related to primary tumor size, tumor grading, disease stage or the presence of carcinoma in the frozen samples.

Sentinel nodes

From 50 out of 58 patients, apparent sentinel nodes were detected and surgically retrieved. This retrieval rate is in line with the surgical learning-curve, of the period during which samples were collected for the present study (28). A total of 94 nodes was obtained from these patients. From 75 nodes of 44 patients, sufficient frozen material was available for qRT-PCR.

Immunostaining: Examination of the above 75 nodes for tumor contamination by means of H&E staining yielded 17 tumor-positive nodes in 14 patients. Immunostaining for EGP-2 and CK19 indicated the presence of tumor in two nodes from two additional patients. Thus, a total of 16 out of 44 patients had tumor-positive sentinel nodes, as indicated either by H&E or immunostaining (19 nodes). One node was tumor-negative with immunostaining, while positive for tumor in one adjacent H&E examined slide. The H&E slide on the opposite side of the part of the node, examined with immunostaining and qRT-PCR, was negative for tumor. This indicates that apparently no residual tumor tissue was present in the part of the node examined with immunostaining and qRT-PCR.

In all 10 control non-cancer lymph nodes, EGP-2 staining was negative, while 9 out of 10 nodes showed occasional CK19 positive dendritic reticulum cells.

QRT-PCR: similar as in the primary tumors, in the sentinel nodes a correlation was found between the mRNA expression levels of EGP-2 and CK19 ($r=0.301$, $p=0.047$). The mRNA expression of both EGP-2 and CK19 was related to the presence of tumor by means of H&E and/or immunostaining: the mean EGP-2 mRNA expression was equivalent to 24,010 (SEM 12,734) MCF-7/10^6 leukocytes in tumor-positive sentinel nodes, and 69 (SEM 34) MCF-7/10^6 leukocytes in tumor-negative nodes ($r=0.361$, $p=0.016$). The mean CK19 mRNA expression was equivalent to 15,704 (SEM 7,669) MCF-7/10^6 leukocytes in tumor-positive sentinel nodes, and 124 (SEM 70) MCF-7/10^6

leukocytes in tumor-negative nodes (r=0.385, p=0.01). Tumor-positive nodes had a higher mRNA expression of EGP-2 (p<0.001) and CK19 (p=0.004) than tumor-negative nodes. The above findings are shown in figure 2.

Three of the 19 sentinel nodes demonstrated to be tumor-positive with H&E and/or immunostaining, showed no/very low EGP-2 or CK19 mRNA expression. One of these nodes was tumor-positive with H&E (on one side) but not with immunostaining; as described above, apparently no residual tumor was left in the part of the node, examined with immunostaining and qRT-PCR. In the other 2 nodes, this may be caused by sampling errors as well, as only very few tumor cells were detected by H&E and immunostaining. The other 16 of the 19 sentinel nodes, demonstrated to be tumor-positive with H&E and/or immunostaining, were all positive with qRT-PCR. In these 16 nodes, tumor presence was observed on both sides of the part examined by immunostaining and qRT-PCR. Numbers are also given in table 2.

Of the remaining sentinel nodes, found tumor-negative with H&E and/or immunostaining, 3 nodes were shown to have mRNA expression of either EGP-2 or CK19 (>2x SD above the mean). All of these 3 nodes contained dendritic reticulum cells, staining positively for CK19 (example shown in figure 3), but no tumor cells.

In none of the control non-breast cancer lymph nodes, EGP-2 or CK19 mRNA expression was detected.

Peripheral blood samples

From 58 patients, 149 blood samples were obtained. At t_0 55, at t_1 54 and at t_2 40 samples were drawn. When t_2 blood samples were not obtained, this was due to the fact that patients were already dismissed from the hospital.

Immunostaining: None of the 149 blood samples, were found to contain tumor cells in the slides for morphology assessment (Giemsa). Immunostaining with antibodies MOC31 and anti-CK19, showed that none of these samples contained EGP-2- or

CK19-positive tumor cells. However, in some samples (n=2), EGP-2-and CK19-positive cell fragments were observed: these were found not to represent (tumor) cells.

QRT-PCR: in a total of 31 samples from 19 patients, EGP-2 or CK19 mRNA expression was found (range EGP-2 expression 1-447 MCF-7/10^6 leukocytes in 16 samples; range CK19 expression 2-1,945 MCF-7/10^6 leukocytes in 15 samples). This is shown in figure 4. At t_0, 13 samples were positive (7 for EGP-2 mRNA expression, and 6 for CK19 mRNA expression), at t_1, 10 samples were positive (5 for EGP-2 and 5 for CK19), and at t_2, 8 samples were positive (4 for EGP-2 and 4 for CK19).

The presence of positive samples for either EGP-2 or CK19 mRNA expression was correlated to CK19 mRNA expression in the sentinel node ($r=0.363$, $p=0.015$) and grading ($r=0.329$, $p=0.014$) of the primary tumor. In 7 patients, more than one consecutive peripheral blood sample was found positive for either EGP-2 or CK19 mRNA expression. The occurrence of more than one qRT-PCR positive blood sample was correlated to sentinel node expression of EGP-2 ($r=0.425$, $p=0.004$) and CK19 mRNA ($r=0.330$, $p=0.029$).

In 4 patients, 1 sample was found with mRNA expression of both EGP-2 and CK19. The occurrence of mRNA expression of both markers in one peripheral blood sample, was again correlated to sentinel node expression of EGP-2 ($r=0.533$, $p<0.001$) and CK19 mRNA ($r=0.406$, $p=0.006$). As in primary tumors and sentinel nodes, the mRNA expression of EGP-2 and CK19 was correlated in these peripheral blood samples ($r=0.794$, $p<0.001$).

DISCUSSION

The possibility to detect micrometastatic disease in breast cancer, is still hampered by the fact that the optimal detection method including a clear marker for detection of minimal amounts of tumor cells, is still lacking. We applied two target markers, and two detection methods, to a series of matching tumor, sentinel node and blood samples of breast cancer patients.

The target markers were EGP-2 and CK19. Both these markers have been described to give false positive results in peripheral blood samples (12, 13, 15). The quantitative RT-PCR method was therefore used to establish a 'cut-off' point to exclude low-level or insignificant EGP-2 or CK19 mRNA expression. With the qRT-PCR, no false- positive results for either marker were observed in control peripheral blood or control lymph nodes in the present study. This important finding is in contrast to the earlier studies and may be explained by the fact that no separate cDNA processing steps were used with this qRT-PCR method, in which RNA is directly used for the analysis. This may reduce possible contamination with exogenous epithelial targets, leading to false positive results (20). It is clear that the real-time evaluation allows the detection of a signal at the earliest stage, providing a more accurate indication of minimal amounts of tumor cells than older RT-PCR methods, that measure at an end-point (9-11, 13). With all samples, the results on mRNA expression of either EGP-2 or CK19 were related to the standard of MCF7 tumor cells diluted in control leukocytes. This was done to allow comparisons of all the analyzed material, and not for speculation on the amount of tumor cells present in the patient sample -as tumor cell lines likely have different (i.e. higher) expression levels than patient tumor cells (20).

In primary tumors, EGP-2 as well as CK19 mRNA expression varied widely ($>10^4$ fold, and $>10^3$ fold respectively). Although the mRNA expression of EGP-2 and CK19 correlated in primary tumors, clearly there are certain tumors that do not have

a simultaneously high or low expression of both markers. Varying mRNA expression levels of tissue specific markers in breast cancers have not been described before. This finding has a number of implications. First, it may be difficult to quantify tumor load by assessing mRNA expression of tumor-associated tissue specific markers, even when mRNA expression levels are quantified. This was already suggested based on varying expressions in cell lines (20), and it can be concluded that the data from the present study support this idea. Second, this finding may question the use of multiple target markers for the detection of micrometastatic breast cancer. Multiple targets were suggested to improve the specificity of PCR based detection (30), compared to the single target mRNA detection methods (traditionally CK19, 9-11, 31-34). However, when not all tumors express multiple targets at similar levels, this approach may actually lead to false-negative results. Therefore, based on the results presented here, we suggest that the sensitivity of multiple-marker assays would likely benefit when based on expression of either one of the targets.

In the sentinel nodes, two extra nodes were found tumor-positive with immunostaining, compared to examination after routine H&E staining. This is in line with conversion rates reported in other studies (35-37). The specificity of qRT-PCR on sentinel nodes was impaired by the observation of 3 false positive nodes. These nodes were found to contain dendritic reticulum cells. These cells have been described to express cytokeratins, and are considered a pitfall in detection of micrometastases using immunostaining (38). Immunostaining allows visual distinction of these cells from tumor cells, but the qRT-PCR method does not. We also observed dendritic reticulum cells in control lymph nodes by CK19 immunostaining, but these few cells were not CK19 positive with qRT-PCR. Furthermore, 3 of 19 nodes (positive for tumor with H&E or immunostaining) were found to be false-negative by qRT-PCR. One node was exceptional because apparently no residual tumor was present (also immunostaining was negative). The other two nodes contained few tumor cells,

detectable with immunostaining, but not by qRT-PCR. The reason is probably that the sections of lymph nodes contained so many 'diluting' lymphocytes that the expression in single tumor cells was below the detection level. This problem could possibly be solved by analyzing each section of the lymph nodes separately, thus minimizing the quantity of analyzed tissue. This is hardly compatible with the purpose of a sensitive, yet practically feasible detection method for micrometastases. Furthermore, these problems are not encountered with the conventional immunostaining. For the sentinel nodes therefore, it may be suggested that a qRT-PCR method such as described here, particularly when using tumor-associated tissue-specific markers, are not specific and sensitive enough for detecting micrometastases. In spite of earlier reports in favor of PCR based detection methods (39), immunostaining appears more suitable for detection of micrometastases in lymphoid tissue in particular (35). This is in line with previous reports by Bostick et al. (12), showing that tumor-associated tissue-specific marker expression in lymph nodes does not necessarily imply the presence of tumor. In view of this, recent reports on the clinical significance of PCR detection of micrometastases from solid tumors in lymph nodes without immunostaining confirmation should be regarded with caution (40, 41), especially with the use of markers with a high propensity of inducing aspecific results (12).

In peripheral blood samples of 33% of patients, EGP-2 or CK19 mRNA expression was observed. Seven patients had more than one positive blood sample, and 4 patients showed a blood sample with simultaneous expression of both EGP-2 and CK19. None of these samples contained immunostaining positive tumor cells. Only a few immunostaining positive cell fragments were found in these samples, although we have previously observed occasional aspecific CK19 staining of segmented granulocytes in peripheral blood samples (unpublished data). Several studies have used only a PCR based method for detection of circulating tumor cells in breast cancer patients (9, 11, 32, 34), but it has been suggested that immunostaining may be

performed as validation for PCR findings in peripheral blood (20). However, a realistic risk of sampling error between PCR and immunostaining samples remains in the 'diluted' setting of single circulating tumor cells, which implies that tumor cells are not necessarily present in both samples. Furthermore, tumor cells with high expressions may still be detected by the qRT-PCR assay, while their number is below the detection limit of the immunostaining method. On the other hand, mRNA expression of tumor-associated tissue-specific markers in peripheral blood does not necessarily imply the presence of tumor (42). Early reports indicated that breast cancer surgery may induce shedding of tumor cells into peripheral blood during operation (43, 44). These data have not yet been confirmed in larger studies. Our present data appear not to support these results, as the incidence of qRT-PCR positive samples was not increased during operation. Our data are roughly in line with other studies combining immunostaining and (q)RT-PCR, in which an incidence of patients with positive peripheral blood samples of 5 to 9% with immunostaining, and 13 to 36% with (q)RT-PCR was reported (11, 32). In metastatic breast cancer patients, the reported incidence of blood samples positive for CK19 mRNA expression by qRT-PCR was up to 50%, and decreased with disease response (33). No previously published data are available on EGP-2 mRNA expression in peripheral blood patient samples.

In conclusion, using a combination of two targets and two techniques in relation to the standard H&E examination, we evaluated the possibilities to detect breast cancer micrometastases. In primary tumors, EGP-2 and CK19 expression was found with a wide variation in expression levels. So, both markers may be used in qRT-PCR for adequate sensitivity of single tumor cell detection. In sentinel nodes, immunostaining appeared more sensitive and specific than H&E staining or qRT-PCR. In peripheral blood, no immunostaining positive samples were found, while many proved to be positive by qRT-PCR. The clinical implications of these findings will have to be clarified in large studies with long-term clinical follow-up data.

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TABLE 1: **Patient and tumor characteristics**

Patients:		
Total no.		58
Mean age (years)		60 (range 35-89)
Disease stage (no. of patients):	I	21
	II a	21
	II b	12
	III a	3
	IV	1
Tumors:		
Total no.		60 (of 57 patients)
Mean size (cm)		2.2 (range 0.8-6)
Histological tumor type:	ductal carcinoma	53
	lobular carcinoma	2
	DCIS	2
	benign	2
	undifferentiated	1
Differentiation grade	I	16
	II	23
	III	15
	not assessable	2
	DCIS (III)	2
	benign	2

TABLE 2: **detection of EGP-2 or CK19 positive cells**

	H&E *	**Immunostaining**		**QRT-PCR**	
		EGP-2	CK19	EGP-2	CK19
Primary tumors (n=52)	46	52	52	52	52
Sentinel nodes (n=75)	17	18 **	18	18	16
Peripheral blood (n=149)	0	0	0	16	15

* positive for malignant tumor cells

** - 1 sample was found tumor-positive with H&E, but negative for immunostaining
and/or qRT-PCR: apparently no residual tumor
- 2 nodes were found tumor-positive with immunostaining additional to evaluation after routine H&E: in total 19 SN were tumor-positive by means of H&E or immunostaining
- of these 19 SN, 3 showed no (1x EGP-2, 3x CK19) or low (2x EGP-2) expression with qRT-PCR

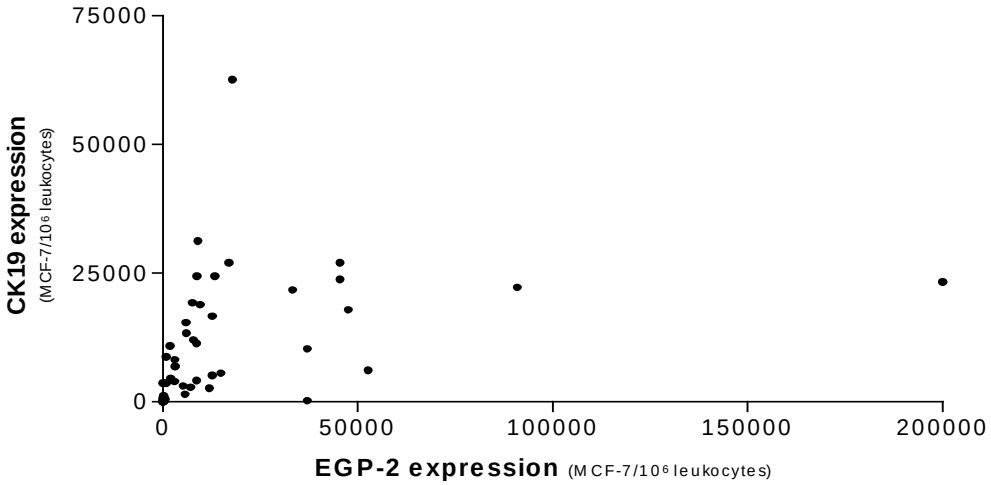

FIGURE 1: Expression of EGP-2 and CK19 mRNA in primary tumors

X-axis: EGP-2 mRNA expression (related to the standard curve of MCF-7/10^6 leukocytes). Y-axis: CK19 mRNA expression (related to the standard curve of MCF-7/10^6 leukocytes).

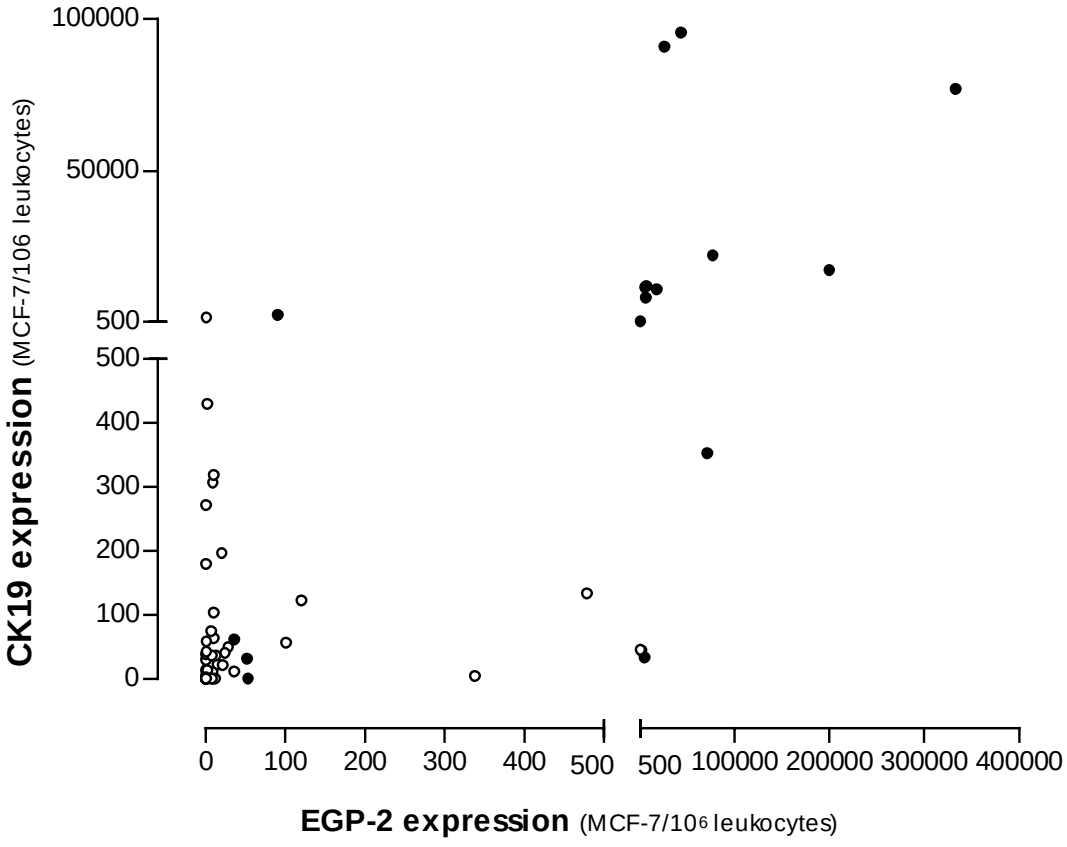

FIGURE 2: Expression of EGP-2 and CK19 mRNA in sentinel nodes

X-axis: EGP-2 mRNA expression (related to the standard curve of MCF-7/10^6 leukocytes). Y-axis: CK19 mRNA expression (related to the standard curve of MCF-

$7/10^6$ leukocytes). The open dots reflect the H&E/immunhistochemical tumor-negative sentinel nodes; the closed dots reflect the H&E/immunohistochemical tumor-positive sentinel nodes. The figure shows the relation between EGP-2 and CK19 expression in sentinel nodes, and the relation between this expression and the presence of tumor in the sentinel nodes.

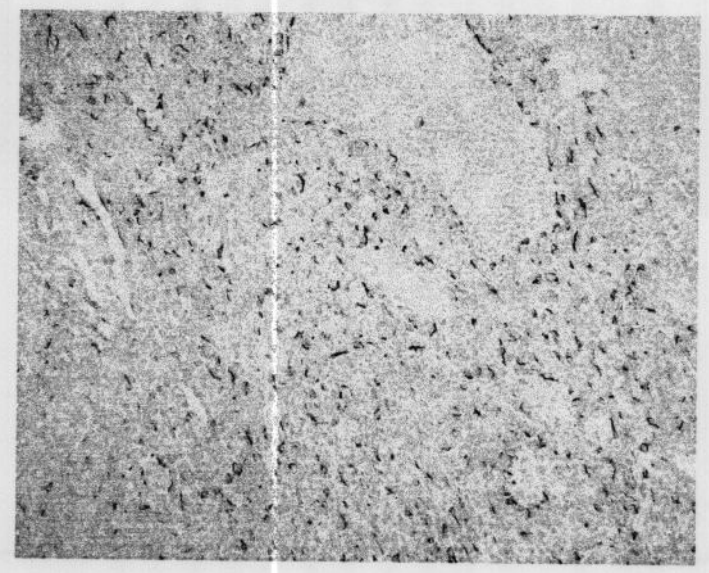

FIGURE 3:

Dendritic reticulum cells in lymph node tissue staining positive for CK19

CK19 positive dendritic reticulum cells in a sentinel node, magnification 100x (reflected as black staining in a furthermore negative lymph node). This sentinel node belonged to a patient who also had a blood sample positive for CK19 mRNA expression.

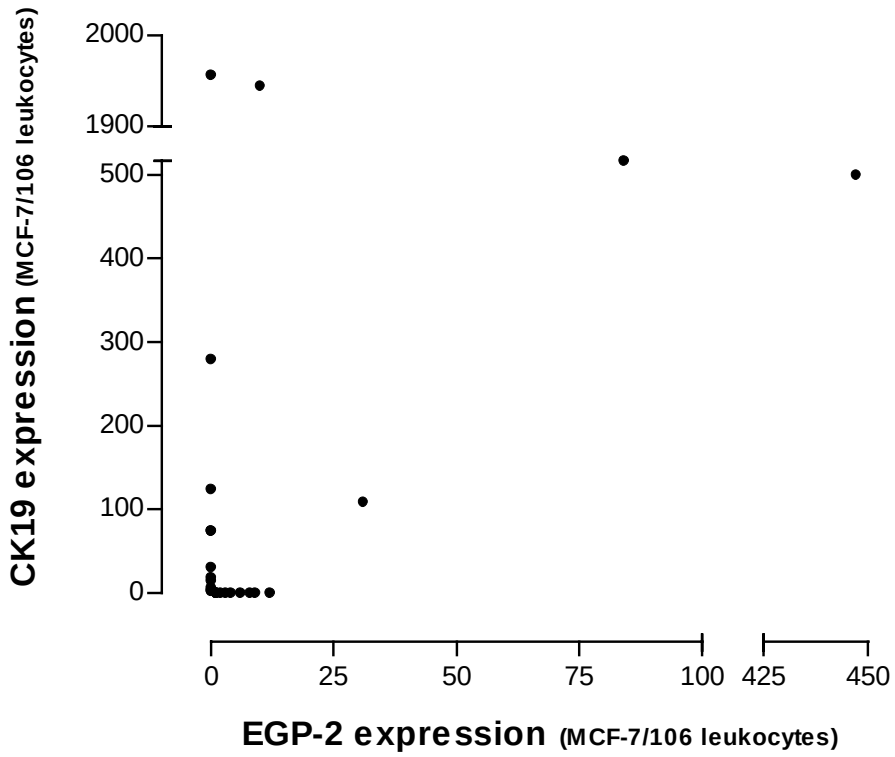

FIGURE 4:

Expression of EGP-2 and CK19 mRNA in peripheral blood samples

X-axis: EGP-2 mRNA expression (related to the standard curve of MCF-7/10^6 leukocytes). Y-axis: CK19 mRNA expression (related to the standard curve of MCF-7/10^6 leukocytes).

Real time quantitative RT-PCR and immunostaining for detection of circulating tumor cells in breast cancer patients

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ABSTRACT

Introduction: the predictive value of conventional staging in breast cancer is limited, and more sensitive staging methods may be useful clinically in selecting patients for adjuvant systemic therapy. The aim of this study was to evaluate detection methods for cells positive for epithelial glycoprotein-2 (EGP-2) and cytokeratin 19 (CK19), using immunostaining and real time quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR). **Patients and methods**: from 59 breast cancer patients, randomized to receive standard- or high-dose chemotherapy and peripheral blood stem cell (PBSC) transplantation, peripheral blood samples (PB, collected prior to, during and after chemotherapy treatment, 145 samples) and PBSC samples (in the high-dose group, 29 samples) were collected. Immunostaining was performed using EGP-2 and CK19 antibodies. Controls were: 11 healthy volunteer PB samples, and 3 PBSC samples from patients with hematologic malignancies. Detection limits were 1 tumor cell of breast cancer cell line MCF-7 in 2.10^6 leukocytes for immunostaining, and 1 MCF-7 in 10^6 leukocytes for qRT-PCR. **Results**: Two PB samples from 2 patients (3%) were tumor positive with EGP-2 immunostaining. Expression was found of EGP-2 mRNA in twelve samples (5 PBSC, 7 PB) from 12 patients (20%), and for CK19 mRNA in one other PB sample (1 patient, 2%). One patient had one immunostaining and a qRT-PCR positive sample, but at different time-points. Controls were negative with both immunostaining and qRT-PCR. **Conclusions**: qRT-PCR and immunostaining with two markers were used for detection of micrometastatic breast cancer in sequential PB and PBSC samples. Of 59 patients, 3% had an immunostaining positive sample, and 22% had a sample positive for EGP-2 or CK19 mRNA expression. The clinical implications of these findings will have to be clarified in large studies with clinical follow-up data.

INTRODUCTION

Staging of breast cancer patients, to determine prognosis and treatment, is largely based on tumor size and axillary node-status. However, the prognostic value of node-positive cancer based on conventional analysis is limited, and 40% of women with tumor positive lymph nodes survive more than 10 years (1) without developing distant metastases. Only part of these women are likely to benefit from adjuvant systemic treatment, and more sensitive staging methods may facilitate the clinician to make a better selection of these patients (2, 3). Particularly in view of the use of adjuvant high-dose treatment, requiring haematopoietic stem cell transplantation to counteract profound bone marrow aplasia, this selection may have clinical impact. In the setting of early adjuvant systemic treatment, the ability to monitor the effects of treatment directly with a known substrate, might lead to improved treatment modalities. Detection of tumor cells in hematopoietic stem cells products may provide information on their clinical impact, in view of stem cell transplantations (3). Therefore, the detection of breast cancer micrometastases (by means of immunohistochemistry or the polymerase chain reaction technique) has gained interest in recent years (4-27).

Conventional immunostaining techniques were described to allow visual confirmation of the actual nature of the stained (tumor) cells, but they appear to be very laborious of nature (2-10). From RT-PCR based methods, targeting tumor-associated tissue-specific antigens, the high sensitivity was recognized early (15-17), but the risk of the risk of giving false positive results soon followed (18-23). In view of this, we examined the value of the detection of micrometastases by means of immunostaining as well as a real time quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) method in peripheral blood and peripheral blood stem cell samples of breast cancer patients. Two epithelial markers were

used: epithelial glycoprotein-2 (EGP-2) and cytokeratin 19 (CK19). EGP-2, also called Ep-CAM, is a 40-kDa membrane-bound glycoprotein. As a pan-epithelial/carcinoma marker, EGP-2 is universally expressed in breast cancer specimens (28), and has been used as a target antigen in a number of carcinoma-directed immunotherapeutical approaches (29-31). Results were compared with tumor cell detection based on the intracellularly located tissue-specific cytokeratin CK19, commonly used for the detection of micrometastases in solid tumors (12, 13). The analyses were performed on a unique series of sequential peripheral blood and stem cell samples from breast cancer patients randomized to receive either standard- or high-dose treatment and stem cell transplantation. The parallel use of these detection methods with two well-known markers for cancer detection, on a large collection of blood and stem cell samples has not been described before. This setting provided the opportunity to study their value for detection of micrometastases in breast cancer patients, before and during standard- or high-dose adjuvant treatment.

PATIENTS AND METHODS

Patients

Patients included in this study participated in a national randomized adjuvant breast carcinoma study (32). Chemotherapy naive breast cancer patients with four or more tumor-involved axillary lymph nodes (stage II and III, according to the TNM system of the Union Internationale Contre le Cancer, 1997), ≤ 55 years of age with negative chest X-ray, liver ultrasound and bone scan, were randomized to receive 5 courses of standard-dose chemotherapy followed by radiotherapy, or 4 courses of the same combination chemotherapy followed by high-dose chemotherapy, peripheral blood stem cell (PBSC) transplantation and radiotherapy. From now on, these groups will be referred to as the standard-dose group, and the high-dose group, respectively. The combination chemotherapy consisted of 5-fluorouracil (500 mg/m^2), epirubicin (90 mg/m^2) and cyclophosphamide (500 mg/m^2), administered intravenously 1x/3 weeks. For the high-dose group, PBSC were mobilized following the third or last course of FEC with daily subcutaneous recombinant human granulocyte-colony stimulating growth factor (rhG-CSF, 263 μg), from day 2 of the course. Leucapheresis was performed from day 9 of this course by means of continuous flow cell separation, until ≥5.10^6 CD34+ cells/kg body weight (as determined by flow cytometric analysis with the fluorescein isothiocyanate-labelled anti-CD34 antibody directed against the HPCA-2 epitope on CD34+ cells, Becton Dickinson, Leiden, the Netherlands) were obtained. High-dose chemotherapy consisted of cyclophosphamide (1,500 mg/m^2), thiotepa (120 mg/m^2) and carboplatin (400 mg/m^2) on days -6, -5, -4 and -3, followed by reinfusion of PBSC on day 0. After reinfusion, daily subcutaneous rhG-CSF was administered until the leukocyte count exceeded 3.10^9/L. Locoregional radiotherapy (50 Gy in 25 fractions) was administered after completion of the chemotherapy scheme with sufficient bone

marrow recovery (defined as platelets >100.10^9/L). Oral tamoxifen 40 mg daily was administered after platelet recovery for at least two years, in both groups. The study, and the collection of blood or PBSC samples as described, was approved by the Medical Ethical Committee of the University Hospital Groningen. All patients, enrolled in the University Hospital Groningen for the national randomized study, were approached to participate for collection of blood samples (and PBSC samples, in the high-dose group) from March 1997 until May 1999. All participating patients gave informed consent.

Sampling times

Sampling times were: t_0: directly prior to start of chemotherapy (peripheral blood); t_1: day 9, 10 or 11 after the third or fourth course of FEC (peripheral blood in the standard-dose group or PBSC material in the high-dose group); t_2: 6 to 8 weeks after completion of chemo- and radiotherapy.

Collection and isolation of nucleated cells from patient samples

Peripheral blood samples (of 40 mL each), and PBSC samples (5 mL) were collected in tubes containing ethylenediamine tetraacetate (EDTA) as anti-coagulant, after prior collection of a waste amount of sample (2 mL). Samples were put on ice immediately. Erylysis was performed with an ammonium chloride solution (155 mM NH_4Cl, 10 mM potassium hydrogen carbonate, 0.1 mM sodium EDTA). The cells to be used for RNA analysis (3/4 of the total amount of cells) were transferred into a guanidinium-isothiocyanate solution (GITC solution: 4 M guanidinium-isothiocyanate, 0.5% n-lauroyl sarcosine, 25 mM sodium-citrate pH 7.0 and 0.1M β-mercaptoethanol) and kept at -20°C, until further processing. Control blood samples were collected from healthy volunteers (laboratory co-workers, after consent), and processed in the same fashion as described above, to be used as negative controls in the qRT-PCR and

immunostaining methods described below. Also, the sensitivity of this qRT-PCR method was determined in control healthy donor leukocytes in which (EGP-2 and CK19 positive) breast cancer cell line MCF-7 tumor cells were spiked in various dilutions. Control PBSC material was obtained from 3 non-breast cancer patients (with a haematological malignancy, after informed consent), and processed in a similar fashion.

The cells to be used for immunostaining (1/4 of the total amount of cells) were allowed to sediment for 1 h onto slides, through a metal funnel-like device, allowing the analysis of 1.10^6 cells on one slide. Cells were then fixed for 10 min at -20°C, in a solution of 4% acetic acid in methanol, followed by 10 min at -20°C, in acetone. Slides were stored at -20°C until further processing. Of each patient sample, one slide was used for Giemsa staining to assess morphology.

Immunostaining

Nucleated cell slides from blood samples were pre-treated with 0.3% hydrogen peroxide for 15 min to block endogenous peroxidase activity of nucleated cells, followed by rinsing with phosphate buffered saline (PBS) solution (0.14 M NaCl, 2.7 mM KCl, 6.4 mM $Na_2HPO_4.2H_2O$, 1.5 mM KH_2PO_4, pH 7.4). At least two of these slides (e.g. 2x 1.10^6 cells) were immunostained with each primary antibody. Samples were stained with the monoclonal antibody directed against EGP-2 (MOC31), using indirect immunoperoxidase staining with horseradish peroxidase conjugated rabbit anti mouse as a secondary antibody (Dako, Glostrup, Denmark) and 3-amino-9-ethylcarbazole as substrate. Samples were counterstained with hematoxylin. The antibody directed against cytokeratin was anti-CK19 (clone 170.2.14, Roche Diagnostics, Almere, The Netherlands), diluted 1:250 on slides in PBS, 1% bovine serum albumin (BSA, Life Technologies, Breda, The Netherlands). Prior to staining with the CK19 antibody, slides were treated with a 0.01% trypsin (Life Technologies,

Breda, The Netherlands) solution in 0.1% $CaCl_2$ in 0.1 M Tris (hydroxymethyl) aminomethane solution, pH 7.8, at 37°C for 5 min, and the indirect immunoperoxidase staining procedure was used as described above.

Isotype specific controls for MOC31 and CK19 were performed using primary antibody mouse IgG1 (X0943, Dako, Glostrup, Denmark). In each staining procedure, a negative control was included using PBS, 1% BSA without the primary antibody, and a positive control prepared from MCF-7 cells on slides. Patient samples were independently examined for morphological features by a technician as well as a pathologist.

In slides containing leukocytes from healthy controls (n=11), no CK19 or MOC31 positive cells were observed. In spiking experiments of MCF-7 cells, diluted in leukocytes, 1 tumor cell could be detected in 2.10^6 leukocytes total, e.g. the total number of cells analyzed.

RNA isolation

RNA isolation from all samples was performed by means of the Rneasy Mini Kit (Qiagen, Westburg b.v., Leusden, The Netherlands), according to the manufacturer's instructions. Briefly, a quantity of cell lysate corresponding with 5.10^6 nucleated cells was obtained. One volume of 70% ethanol was added to the lysate, and mixed well by pipetting. The mixture was applied to a spin column, to allow adsorption of RNA to the membrane, and the column was subsequently centrifuged at 8,000 g, for 30 sec. Columns were washed and the RNA sample was eluted from the column by means of RNase free water, after centrifugation at 8,000 g for 1 min, and maintained in 50 µL Rnase free water.

The integrity of the RNA samples was assessed on formaldehyde-containing (2.2 M) agarose gels. Only RNA samples with visible and discrete 28S and 18S ribosomal RNA bands were used for the RT-PCR experiments.

Quantitative RT-PCR (qRT-PCR) for assessing EGP-2 and CK19 expression

The qRT-PCR was performed by means of the LightCycler (Roche Diagnostics, Mannheim, Germany), according to the manufacturer's instructions. The LightCycler system is based on the continuous monitoring of the formation of PCR product by measuring the amount of hybridization probes annealing to the target sequence in every cycle. Hybridization probes emit a fluorescent signal, that correlates to the concentration of target. The sequence of specific primers and probes was checked prior to use to avoid amplification of genomic DNA or

pseudogenes (CK19), by means of software packages SeqMan 3.57 and PrimerSelect 3.10 (DNASTAR Ltd., London, U.K.). A volume of 5 µL total RNA in 20 µL master mix (Roche Diagnostics, Mannheim, Germany), 3 pmol of the specific primers and 2 pmol of the specific hybridization probes was added to each glass capillary. The sequences of the EGP-2 specific primers were: EX3F: 5'-GAACAAT-GATGGGCTTTATG-3' (corresponding to bases 374 to 394 of the EGP-2 cDNA) and EX7R: 5'-TGAGAATTCAGGTGCTTTTT-3' (bases 868 to 888). The sequences of the EGP-2 specific probes were: ALL-FL: ATCCAGTTGATAACGCGTTGTGAT-x and ALL-NEW: Red 640-TCCTTCTGAAGTGCAGTCCGCAA-p. The sequence of the CK19 specific primers were: CK19 F: 5'-ACTACAGCCACTACTACACGAC-3' (corresponding to bases 409-430), and CK19 R: CAGAGCCTGTTCCGTCTCAAAC (corresponding to bases 557-536). The sequences of the CK19 specific probes were: CK19-FL: TGTCCTGCAGATCGACAACGCCC-x (corresponding to bases 485-507), and CK19-705: Red 705-TCTGGCTGCAGATGACTTCCGAACCA-p (corresponding to bases 509-534). The detection of EGP-2 or CK19 target was performed in separate glass capillaries.

Samples were submitted to an initial reverse transcriptase hybridization step of 25 min at 55°C, followed by a denaturation step of 30s at 95°C, then amplification in a three-step cycle procedure (denaturation 95°C, ramp rate 20°C/s; annealing 56°C, 15s, ramp rate 20°C/s; and extension 72°C, 20s, ramp rate 2°C/s) for 45 cycles. Finally, a melting three step cycle was performed (95°C, ramp rate 20°C/s; 60°C, 10s, ramp rate 20°C/s; 95°C, ramp rate 0.2°C). On several occasions, after the LightCycler procedure, the contents of the glass capillaries were also checked on gel, always conveying the expected PCR product for the specific primers in tumor positive samples. In all runs, a standard curve was obtained using the same samples of RNA from dilutions of MCF-7 cells in healthy donor leukocytes (1:10; $1:10^2$; $1:10^3$; $1:10^4$; $1:10^5$; $1:10^6$ respectively), with a total of 1.10^6 cells. All

results were always expressed in relation to the standard curve. Corrections were made for the expression of β-2 microglobulin. The sequence of the β-2 microglobulin specific primers were: β-2 microglobulin F: CCAGCAGAGAATGGAAAGTC (corresponding with bases 33 to 52) and β-2 microglobulin R: GATGCTGCTTACATGTCTCG (corresponding with bases 301-282). The sequences of the β-2 microglobulin specific probes were: β-2 microglobulin-FL: TTCTTCAGTAAGTCAACTTCAATGTCGGA-x (corresponding to bases 118-90) and β-2 microglobulin-705: LC Red 705-ATGAAACCCAGACACATAGCAATTCAG -p (corresponding to bases 86-60). As a positive control, the RNA from undiluted MCF-7 cells was used; as a negative control, a sample was used to which no RNA at all was added. Also in each experiment, a sample containing healthy donor leukocytes without tumor cells was included as a negative control.

With this method, a sensitivity was obtained of 1 spiked MCF-7 cell, detected in 1.10^6 healthy donor leukocytes (n=3 separate experiments). In leukocyte samples from n=11 healthy donors, the EGP-2 or CK19 signal remained below this detection limit.

Statistics

Statistical analyses were performed by means of the SPSS statistical software package. The relations between PCR or immunostaining and tumor size, number of tumor positive lymph nodes, the presence of tumor in the apical lymph node, and tumor characteristics were analyzed by means of the Pearson correlation test. Only p-values <0.05 were considered significant.

RESULTS

Patients

A total of 59 breast cancer patients participated (of n=60 eligible patients) in this study from March 1997 until May 1999. The standard-dose group consisted of 29 patients, and the high-dose group of 30 patients. Mean age at the start of treatment was 45.1 years (range 28-56 years) and 45.7 years (range 33-54 years) in these groups respectively (N.S.). Patient characteristics are reflected in table 1. A total of 174 samples were collected (59 at t_0, 57 at t_1 of which 29 PBSC samples, and 58 at t_2).

Immunostaining

None of the 174 blood samples, were found to contain tumor cells in the nucleated cell slides for morphology assessment (Giemsa). With immunostaining, nucleated cell slides from blood samples were found to contain a cell staining positive with MOC31, identified as being a tumor cell, in two cases: one patient of the standard-dose group at t_1, and one patient of the high-dose group at t_1 (PBSC material). Therefore, 2 patients out of 59 total (3%) had a tumor positive sample by immunostaining. In none of the other samples immunostaining positive (tumor) cells or cell fragments were identified, neither with MOC31 or the antiCK19 antibody.

Real time qRT-PCR for the expression of EGP-2 and CK19

An overview of samples positive for immunostaining or qRT-PCR is given in table 2. In a total of 12 patient samples of 12 patients, EGP-2 mRNA expression was found: in the standard-dose group, at t_0 (1 peripheral blood sample: expression equivalent to 28 MCF-7/10^6 leukocytes) and t_2 (3 peripheral blood samples: expressions equivalent to 15 to 17 MCF-7/10^6 leukocytes). In the high-dose group, EGP-2

mRNA expression was found at t_1 (5 PBSC samples: expressions equivalent from 15 to 29 MCF-7/10^6 leukocytes), and at t_2 (3 peripheral blood samples: expressions equivalent from 14 to 29 MCF-7/10^6 leukocytes). In one peripheral blood sample, CK19 mRNA expression was found: in the standard-dose group, at t_0. The expression in this sample was equivalent to 10 MCF-7/10^6 leukocytes of the standard. Thus, of 29 PBSC samples, 5 were qRT-PCR positive and 1 was immunostaining positive, while of 145 peripheral blood samples, 8 were positive with qRT-PCR and 1 with immunostaining.

The peripheral blood sample positive for CK19 mRNA expression was not found positive for EGP-2 expression. One peripheral blood sample found positive for EGP-2 mRNA expression (standard-dose group, t_0), belonged to a patient with a peripheral blood sample at t_1, containing an EGP-2 positive tumor cell with immunostaining (figure 1). Thus, a total of 13 patients out of 59 (22%) had a sample positive for either EGP-2 or CK19 mRNA expression. However, none of the samples found positive for EGP-2 or CK19 mRNA expression were positive with immunostaining.

No relations between the mRNA expression of EGP-2 or CK19 and tumor size or number of tumor positive lymph nodes was found, except that EGP-2 mRNA expression at t_2 was positively correlated to primary tumor size ($r=0.409$, $p=0.001$). The follow-up ranging from 40 to 12 months after finishing chemotherapy, was considered too modest to allow relating follow-up data to immunostaining or qRT-PCR data.

DISCUSSION

In this study, the detection of micrometastatic breast cancer was explored, by means of a qRT-PCR method, as well as immunostaining. Two markers were used: EGP-2 and CK19. The analyses were performed on a series of sequential samples of blood as well as stem cell material, of breast cancer patients receiving either adjuvant standard- or high-dose chemotherapy including haematopoietic stem cell transplantation. Detection of circulating tumor cells with two markers, by means of immunostaining and qRT-PCR, in this setting has not been described before. In many studies, techniques for detection of micrometastatic disease have been examined (4-27), but as yet in solid tumors, no optimal technique is available. Conventional immunostaining techniques are now acknowledged for allowing visual confirmation of the actual nature of the stained (tumor) cell, but they may be susceptible to inter-observer differences (33). PCR based methods are found to harbor the risk of false positive results with the use of tumor-associated, tissue-specific markers in solid tumors (18-23). In view of this, we chose to combine these two methods in this study. For the detection of circulating tumor cells, in some studies used only a PCR based method was used (15, 21, 25, 27), while this methods was combined with immunostaining in other studies (16, 23, 24, 26),

In addition, we evaluated the presence of both EGP-2 and CK19 positive cells. CK19 is the most commonly used marker for the detection of micrometastatic breast cancer (12, 13), and as such it was used to compare with EGP-2. Both markers can give false positive results, as described in the evaluation of peripheral blood (19, 21, 23). Therefore, we used the qRT-PCR method to quantify expression levels, in order to establish a ‘cut-off’ point to exclude low-level or insignificant EGP-2 or CK19 mRNA expression. No positive results for either marker were observed in peripheral blood from healthy controls in the present study. This important finding

is in contrast to the earlier studies and may be explained by the fact that no separate cDNA processing steps were used with the present qRT-PCR method, in which RNA is directly used for the analysis. This may reduce the risk of contamination with external epithelial targets. This contamination is known to induce false positive results in the setting of micrometastases detection in breast cancer (33, 34). Furthermore, it is clear that the real time evaluation allows the detection of a signal at the earliest stage, providing a more accurate indication of minimal amounts of tumor cells than the older RT-PCR methods, that measure at an end-point (15-17, 19). With all samples, the results on mRNA expression of either EGP-2 or CK19 were related to the standard of MCF7 cells diluted in control leukocytes. This was done, not for speculation on the amount of tumor cells present in the patient sample (as tumor cell lines likely have higher expression levels than patient tumor cells (33), but solely for comparisons of all the analyzed materials. The isolation of the nucleated cell fraction was performed by means of lysis of erythrocytes. This method was described to preserve tumor cells in a fashion superior compared to the frequently used Ficoll isolation (35).

Most studies on molecular detection of micrometastases have relied on a single mRNA marker, usually CK19 (15-17, 24-27). The use of multiple-marker assays for solid tumors has been described before (36, 37) including breast cancer micrometastases (38, 39). One advantage of the use of more than one marker may be the improved specificity of molecular detection methods. The expression of more than one target gene is usually not found in control tissue (39), and may be related to poor prognostic clinico-pathologic factors (38). In view of the fact that, similar as in tumor cell lines (11), the expression of tumor-associated tissue specific markers in primary tumors can vary considerably (unpublished data), it can be postulated that not all tumors are likely to express all the selected markers. It may therefore be considered preferable to use multiple-marker assays to improve detection sensitivity

rather than specificity, by selecting for expression of at least one marker (40). The clinical justification for either one of these approaches remains to be examined. In this study, a total of 15 samples of 14 patients had evidence for the presence of EGP-2 or CK19 positive cells. Two samples were positive with immunostaining, and 13 samples showed either EGP-2 or CK19 mRNA expression. None of the samples showed expression of both markers at the same time, or were positive for mRNA expression as well as immunostaining. In this case therefore, if the aim of using the two markers would be to improve specificity of the qRT-PCR, none of the samples could be regarded as positive. With the opposite approach, to improve sensitivity, 13 samples would be regarded as positive. It has been suggested that recruitment of tumor cells into peripheral blood cannot be confirmed by RT-PCR alone, and that immunostaining should be performed as validation (33). In our study, the application of this suggestion implies that again, none of the 13 samples, positive for mRNA expression, would be considered tumor positive. While this approach is likely valuable in detecting micrometastases in lymph nodes, the setting of tumor cells in the circulation harbors the realistic risk of sampling error between PCR and immunostaining samples, which implies that tumor cells are not necessarily present in both samples. Of 59 patients in this study, 3% had a tumor positive sample by immunostaining (both for EGP-2), and 22% had a sample positive for EGP-2 or CK19 mRNA expression (2% for CK19, and 20% for EGP-2). Most EGP-2 mRNA positive samples were observed at t_1, in the PBSC samples of the high-dose group. In an early report by Brügger et al. (3), it was suggested that tumor cells, detected by CK19 immunostaining, were possibly mobilized into the peripheral blood simultaneously with haematopoietic stem cells. In this study, one PBSC sample was found positive for tumor cell presence with immunostaining for EGP-2, but not for CK19. The EGP-2 mRNA expression in the PBSC samples of 5 patients (a considerable part of patients in the high-dose group: 17%) may originate from the

presence of tumor cells. The high incidence of qRT-PCR positivity in PBSC compared to peripheral blood samples, may in part be explained by the fact that PBSC material already contained relatively more mononuclear cells compared to peripheral blood, due to the leucapheresis selection procedure (31). Whether haematopoietic growth factor G-CSF, used for obtaining PBSC, may induce EGP-2 mRNA expression (as described for tissue specific marker CEA, 41), is as yet unknown.

The patients in this study all had node positive breast cancer, but no distant metastases. In other studies with breast cancer patients of comparable disease stages, the incidence of positive peripheral blood samples varied with immunostaining from 5 to 9% (16, 26) and 13 to 36% with (q)RT-PCR (16, 25-27). In a majority of metastatic breast cancer patients, Smith et al. (24) could monitor disease response using a qRT-PCR for CK19 expression. No previously published data are available on EGP-2 mRNA expression in peripheral blood patient samples. Notwithstanding differences in techniques, our results appear to be in line with those previously published. Like others, we find a lower incidence of tumor cell positive samples with immunostaining than with the qRT-PCR. The clinical implications of this however, are not yet clear.

In conclusion, the use of qRT-PCR and immunostaining for detection of micrometastatic breast cancer disease was evaluated in a series of sequential peripheral blood samples and peripheral blood stem cell material. Of 59 patients, 3% had an immunostaining positive sample, and 22% had a sample positive for EGP-2 or CK19 mRNA expression. The clinical implications of these findings will have to be clarified in larger studies with long-term clinical follow-up data.

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TABLE 1: **Patient characteristics**

		Standard-dose (n=29)	High-dose (n=30)
Age (years)		45.1 (28-56)	45.7 (33-54)
Stage	IIA	9	5
	IIB	18	18
	IIIA	2	7
LN*	< 10	19	19
	≥ 10	10	11
Tumor size (cm)		2.7 (SD 1.4)	3.9 (SD 2.9)

* lymph nodes containing tumor as assessed by standard morphological examination

TABLE 2: **positive samples with immunostaining or qRT-PCR**

		Immunostaining		**QRT-PCR**	
		EGP-2	CK19	EGP-2	CK19
T_0	standard-dose	0	0	1 *	1
	high-dose	0	0	0	0
T_1	standard-dose	1 *	0	0	0
	high-dose	1 +	0	5 +	0
T_2	standard-dose	0	0	3	0
	high-dose	0	0	3	0

* one patient from the standard-dose group had a blood sample positive at t_0 for EGP-2 mRNA expression, as well as an immunostaining positive sample at t_1.

+ The samples of the high-dose group at t_1 were PBSC samples

Purging of epithelial tumor cells from peripheral blood stem cells by means of the bispecific antibody BIS-1

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Abstract

Peripheral blood stem cell (PBSC) support in breast cancer patients allows high-dose chemotherapy, but tumor cell contamination of the PBSC is a potential source of relapse. Specific carcinoma cell kill can be obtained by retargeting activated T cells with bispecific antibody BIS-1, directed against epithelial glycoprotein-2 and CD3. To purge epithelial tumor cells from breast cancer patients PBSC, activation of T cells in PBSC and T cell retargeting by BIS-1 was studied.

PBSC, obtained by leucapheresis after chemotherapy and rhG-CSF, were cultured in the presence of PBS, IL-2, OKT3 or IL-2/OKT3 for induction of T cell activation. Subsequently, lysis of epithelial tumor cell lines by activated T cells of PBSC in the presence or absence of BIS-1, was assessed with the Cr^{51} release assay or immunocytochemical staining. The effect on PBSC hematopoietic colony formation (HCF) was evaluated by the CFU-GM assay.

Prior to activation, breast cancer patients PBSC contained higher levels of CD8+ T cells, compared to peripheral blood from healthy volunteers ($p<0.05$). The potential of PBSC to sustain tumor cell lysis was increased after all prior activations, and was further enhanced by BIS-1. Maximal BIS-1 effect was observed after 72 h OKT3 activation of PBSC ($p<0.0005$), inducing a >3 log depletion of tumor cells. HCF was not affected by prior OKT3 activation, and/or BIS-1.

In conclusion, specific tumor cell lysis by PBSC can be obtained in vitro by OKT3 activation and BIS-1 retargeting of T cells, without affecting HCF. Current studies evaluate this format for future clinical application.

Introduction

Peripheral blood stem cell (PBSC) support in breast cancer patients allows high-dose chemotherapy, but tumor cell contamination is a potential source of relapse as was demonstrated in marker-gene studies in hematological and solid tumor types (1,2). A number f methods to clear tumor cells from PBSC (purging) has been described, including depletion of tumor cells and selection of stem cells from the graft (3,4). Tumor cell depletion by means of treatment with non-selective chemotherapeutical drugs was proven to eliminate tumor cells, but hematopoietic colony formation was negatively affected as well (5). Stem cells selected through enrichment of CD34 positive cells, still contained a number of tumor cells (6).

To obtain a more specific way to eliminate tumor cells from PBSC, treatment with monoclonal antibodies has been studied. The use of antibodies was found to be effective and feasible in purging tumor cells from PBSC in hematological cancer patient studies (7-12), although the sole binding of a monoclonal antibody does not induce tumor cell lysis. In the systemic treatment of solid tumors, antibody-based treatment was shown to be beneficial in a setting of minimal residual disease (13). Compared to former disappointing anti-tumor effects of immunotherapy in patients with high tumor load (14-16), adjuvant administration of monoclonal antibodies was found to induce a survival benefit in colorectal carcinoma patients (17). Immunotherapy gained new interest as also a clinically beneficial effect was seen in disseminated breast cancer patients treated with the humanized anti-HER2 antibody Herceptin™ (18). However, only a minority of patients is eligible for this type of treatment, as HER2/neu expression in breast cancer is around 25-30%. Elimination of breast cancer cells from bone marrow after antigen-binding by means of immunobeads and immunotoxins was shown to be effective in vitro (19, 20).

To increase cytotoxicity, also the use of cytokines has been studied. Interleukin-2 (IL-2) incubation of PBSC induced up to 50% tumor cell kill in vitro (21), and it did not negatively affect stem cell engraftment in breast cancer patients (22). Additional effect of anti-CD3 antibody OKT3, next to IL-2, on tumor cell kill was seen in bone marrow of hematological patients (23). Also in the hematological setting, Kaneko et al. described activation of peripheral blood mononuclear cells with IL-2 and OKT3, combined with bispecific antibodies, for ex-vivo purging of leukemic cells from bone marrow. Adding bispecific antibodies clearly increased cytolysis in this study (24). A bispecific antibody combines affinity to both target and cytotoxic effector cells, thus allowing more efficient cell lysis than with a monoclonal antibody alone (25).

In view of the above, it seems reasonable to further evaluate the combination of activation of T cells present in breast cancer patient PBSC harvests and a bispecific antibody for purging of carcinoma cells from PBSC, which to our knowledge has not been described before. In our study, we have used the bispecific monoclonal antibody BIS-1, which is directed against the pan-carcinoma-associated membrane antigen epithelial glycoprotein-2 (EGP-2) and the CD3 complex present on T cells. EGP-2, also called Ep-CAM, is a 40-kDa membrane-bound glycoprotein, strongly expressed by most carcinomas and universally expressed in breast cancer specimens (reviewed in 26). As such, EGP-2 is a commonly used target antigen in many carcinoma-directed immunotherapeutical approaches (17, 25, 26). The bispecific antibody BIS-1 creates functional cross-linking of the activated T cells and EGP-2 positive tumor cells allowing the delivery of a tumor cell specific lethal hit, and this T cell retargeting with BIS-1 induces specific epithelial tumor cell kill in vitro and in vivo (14, 27). The goal of this study was to examine in vitro activation of T cells present in PBSC harvests obtained from breast cancer patients for generation of cytotoxic effector cells, and to

study purging of epithelial tumor cells from PBSC by BIS-1 retargeting of activated PBSC.

Materials and methods

PBSC (effector cells)

Patients participated in a national randomized adjuvant breast carcinoma study (28), which was approved by the Medical Ethical Committee of the University Hospital Groningen. All patients gave informed consent. As part of this study, PBSC were mobilized after combination chemotherapy (5-fluorouracil, epirubicin and cyclophosphamide, FEC) and recombinant human granulocyte-colony stimulating growth factor (rhG-CSF), and collected by means of leucapheresis apparatus Cobe Spectra™ (Cobe Netherlands, Uden, The Netherlands). Briefly, from day 2 of the third course of FEC, s.c. rhG-CSF 263 μg was administered daily. On day 9, leucapheresis was started by means of continuous flow cell separation. The PBSC harvest consisted of a (nearly granulocyte free) mononucleated cell product. Usually two to three leucapheresis procedures were required until at least 6.10^6 CD34+ cells/kg body weight were collected. PBSC samples for this study were cryopreserved in 10% dimethyl sulfoxide in a maximal final cell concentration of 200.10^6 cells/mL and stored in liquid nitrogen. Prior to experiments, PBSC were thawed rapidly, washed in newborn calf serum (NCS, Gibco Europe, Breda, The Netherlands), incubated for 15 min in 6 mL NCS to which 2000 U DNAse I (Boehringer Mannheim), 0.2 mM magnesium sulfate and 1000 U heparin was added, and centrifuged 5 min at 591 g. Erylysis was performed on all samples (including whole blood control samples) with an ammonium chloride solution (155 mM NH_4Cl, 10 mM potassium hydrogen

carbonate, 0.1 mM sodium ethylene diamine tetra acetate, EDTA). Cells were washed in RPMI medium (Boehringer Ingelheim) and resuspended in RPMI medium containing 5% heat inactivated human pooled serum (HPS), 60 μg/mL gentamycin (Biowithaker, Verviers, Belgium) and 2 mM glutamin, to a final concentration of 1.10^6 nucleated cells/mL.

Activation

PBSC were incubated for 0 h, 24 h and 72 h in the above described culture medium containing one of the following additives: 1) Phosphate buffered saline (PBS) solution (0.14 M NaCl, 2.7 mM KCl, 6.4 mM $Na_2HPO_4.2H_2O$, 1.5 mM KH_2PO_4), pH 7.4, or 2) 100 U/mL IL-2 (aldesleukin, Chiron, Amsterdam, The Netherlands), or 3) 5 %v/v anti-CD3, (tissue culture supernatant containing 10 μg mL^{-1} OKT3), or 4) 100 U/mL IL-2 and 5 %v/v OKT3. Prior to further use, cells were washed in the culture medium without activating additives.

Flow cytometry

After PBSC activation as described above phenotyping of T cells was assessed using: fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-labelled anti-CD4, anti-CD8, anti-CD25, anti-CD69 and anti-HLA DR monoclonal antibodies (Immuno Quality Products, Groningen, The Netherlands). PBSC were incubated for 30 min at 4°C (5 μl antibody for 1.10^6 cells in 100 μL PBS with 1% HPS), washed once in PBS, resuspended in 150 μL PBS. Samples were analyzed on a Coulter Elite Cytometer (Coulter Electronics, Hilaleah, FL) using an argon laser (488 nm) for FITC and PE excitation.

Target cell lines

GLC-1 (EGP-2-negative parental cell line) and GLC-1M13 (EGP-2-positive subclone) are small-cell lung cancer (SCLC)-derived cell lines (29). These cell lines were cultured according to routine procedures in RPMI 1640 based medium supplemented with 14% heat inactivated fetal calf serum (FCS), 2 mM glutamine, 60 μg/mL gentamycin, 0.05 mM β-mercaptoethanol and 1 mM sodium pyruvate at 37°C in a humidified atmosphere containing 5% carbon dioxide (CO_2). The EGP-2 positive (GLC1M13) and EGP-2 negative (GLC1) cell model of similar origin was used in the ^{51}Cr-release assay. For morphological reasons, the EGP-2 positive breast cancer derived cell line MCF-7 was used in the log depletion assay. MCF-7 was cultured according to routine procedures in RPMI based medium supplemented with 10% FCS, at 37°C in a humidified atmosphere containing 5% CO_2.

BIS-1

The BIS-1-producing quadroma was made in our department by fusion of the hybridomas RIV-9 and MOC-31, producing anti-CD3 (IgG3) and anti-EGP-2 (IgG1) antibodies respectively, according to De Lau et al (30). Preparation and purification was performed as described earlier (14). Briefly, BIS-1 was produced on large scale by means of a hollow fiber culture system (Endotronics, Minneapolis, MN). Purification of the hybrid antibodies (IgG3/IgG1) from parental-type antibodies, also produced by the quadroma, was performed by protein A column chromatography. BIS-1 $F(ab')_2$ was then produced by means of digestion by pepsin followed by G150 Sephadex gel filtration, and added to a 0.9% sodium chloride solution to obtain a final concentration of 0.2 mg/mL.

^{51}Cr-release assay

^{51}Cr-release assays were performed according to standard procedures to assess BIS-1 redirected T cell cytotoxicity (14). All determinations were executed in triplicate. 5.10^6 target cells (tumor cells GLC-1 or GLC-1M13) were suspended in 100 µL of culture medium containing 3.7 MBq of [^{51}Cr]sodium chromate (Amersham Pharmacia Biotech Benelux, Roosendaal, The Netherlands) and incubated for 1 h at 37°C in a humidified, 5% CO_2-containing atmosphere. Unbound [^{51}Cr]sodium chromate was removed by washing the tumor cells three times with medium. Subsequently, aliquots of 100 µL medium containing 0, $2.5.10^3$, $2.5.10^4$ or $2.5.10^5$ of PBSC (effector cells) after the above mentioned 24 h or 72 h with PBS, IL-2, OKT-3 or IL-2/OKT3, were added into a 96-well round bottom microtiter plate (Greiner no. 650180, Greiner, Alphen aan de Rijn, The Netherlands). To each well, also 50 µL medium was added containing $2.5.10^3$ [^{51}Cr] labelled target tumor cells, resulting in effector:target ratios of 0, 1, 10 and 100 in a final volume of 200 µL/well. Finally, a 50 µL aliquot of medium with 0.4 µg/mL BIS-1 F(ab')$_2$ (with a final concentration during the assay of 0.1 µg/mL) or 50 µ L medium without BIS-1, was pipetted. The microtiter plates were centrifuged at 46 g, for 2 min and incubated at 37°C in 5% CO_2. After a 4 h incubation, the plates were centrifuged at 182 g, for 5 min and 100 µL samples taken from the supernatant were counted in a γ counter (Wizard, EG&G/Wallac). Cell lysis was calculated from the percentage [^{51}Cr] released, according to the formula: experimental release-spontaneous release divided by maximal release-spontaneous release x 100%. Maximal release was determined from a sample to which 100 µL of 2% Triton X-100 solution was added instead of effector

cells. Spontaneous release was determined from a sample to which 100 μL of medium was added instead of effector cells.

Hematopoietic colony formation

Toxicity of prior T cell activation and subsequent BIS-1 treatment on hematopoietic stem cell recovery was studied with the granulocyte and macrophage-colony forming unit (CFU-GM) assay (31). Briefly, hematopoietic colony formation was assessed in 1 mL Dulbecco's Modified Eagle Medium (DMEM) including 1.1% methyl cellulose, 20% FCS, 1% deionized bovine serum albumin, 1.10^{-3}% α-thioglycerol, and 10 ng/mL IL-3 and GM-CSF. PBSC (2.10^5 cells, after the prior activations as mentioned above) were plated after 4 h incubation with or without BIS-1 (0.1 μg in 200 μL DMEM); with or without GLC1M13 at effector:target ratio 100:1. Cells were plated in 35 mm dishes and cultured for 14 days at 37°C. Hematopoietic colonies containing ≥ 40 cells were counted under an inverse microscope.

Log-depletion assay

To assess the log-depletion of tumor cells by activated PBSC, MCF-7 tumor cells were added to PBSC after 72 h prior activation with OKT3, under conditions as mentioned above. MCF-7 tumor (target) cells were added to (effector) PBSC in an effector: target ratio of 1.10^4:1, in a total volume of 6 mL of RPMI 1640 medium (supplemented with HPS, gentamycin and glutamin, as mentioned above), in the presence or absence of BIS1 (with a final concentration during the assay of 0.1 μg/mL). As a control, MCF-7 tumor cells were also added to 6 mL of RPMI 1640 medium without PBSC. After a 4 h incubation at 37°C in 5% CO, sedimentation of cells unto slides was performed. Cells

were stained with monoclonal antibody MOC31, directed against EGP-2, using indirect immunoperoxidase staining with horseradish peroxidase conjugated rabbit anti mouse as a second antibody and AEC as a substrate. Slides were routineously counterstained with hematoxilin-eosin.

Statistics

Cytotoxic cell lysis, hematopoietic colony formation and leukocyte phenotype were analyzed by means of the Student's t-test. A $p<0.05$ was considered significant.

Results

Cytotoxic activity in PBSC

^{51}Cr-release assay

To purge epithelial tumor cells from PBSC, we studied prior activation of T cells present in breast cancer patient PBSC harvests, combined with BIS-1 in the ^{51}Cr-release assay. Therefore, in vitro tumor cells were added to PBSC harvests after prior T cell activation, in the presence or absence of BIS-1. The effect of BIS-1, and prior activation of PBSC on GLC1M13 (EGP-2 positive) tumor cell lysis is shown in figure 1. Tumor cell lysis was increased by the addition of BIS-1, after all prior activations, compared to cell lysis without BIS-1. Maximal effect of BIS-1 was seen after 72 h of prior PBSC activation with OKT3 ($p<0.0005$ compared to without BIS-1). Tumor cell lysis in the presence of BIS-1 was not significantly different after prior PBSC activation with IL-2/OKT3 compared to OKT3 alone. Addition of BIS-1 did not increase lysis of control GLC1 (the EGP-2 negative counterpart of GLC1M13, and therefore incapable of binding BIS-1), compared to tumor cell lysis without BIS-1 (not shown).

Prior PBSC activation with IL-2, IL-2/OKT3 or OKT3 alone, but without subsequent BIS-1, increased GLC1M13 as well as GLC1 tumor cell lysis also in the absence of BIS-1, when compared to the PBS control (maximum GLC1M13 lysis: $p<0.0005$ compared to PBS after 72 h PBSC activation with IL-2).

Tumor cell lysis of GLC1M13 in the presence of BIS-1 was increased nearly 100% after 72 h of PBSC activation compared to 24 h activation with OKT3 and IL-2/OKT3 ($p<0.005$ and $p<0.025$, respectively).

In figure 2, the effect of increasing ratios of effector: tumor cells is shown. After all PBSC activations, increasing E:T ratio coincided with increased BIS-1 redirected

cytotoxicity (maximal GLC1M13 lysis after OKT3 activation, $p<0.0005$).

Log depletion assay

At an effector: target ratio of 1.10^4 OKT3 activated PBSC to 1 MCF-7 tumor cell, in the presence of BIS-1, a >3 log depletion of MCF-7 tumor cells (mean 0.09% of total number of MCF-7 cells remaining) was observed, as compared to a control to which no effector PBSC was added (see also figure 3). In the absence of BIS-1, only >1 log depletion of MCF-7 tumor cells (mean 6% MCF-7 cells remaining) was observed with OKT3 activated PBSC. The sensitivity of tumor cell detection in these experiments was 1 MCF-7 tumor cell in the total number (e.g. 6.10^7) of PBSC screened, in line with refs. 32 and 33.

Composition of PBSC and activation markers on T lymphocytes

During the three consecutive days of the leucapheresis procedure (day 9, 10 and 11 since chemotherapy), in the PBSC harvest the percentage of CD34+ cells increased ($p<0.05$) and lymphocyte levels decreased ($p<0.05$), but within the lymphocyte compartment the percentage of CD4+ and CD8+ T cells remained the same. The lymphocyte percentage CD8+ T cells in PBSC harvests before activation (mean 28% CD8+ T cells, SD 10%; n=4), was higher compared to peripheral blood of healthy volunteers (15%, SD 1.6%; n=4, $p<0.05$). The lymphocyte percentage of CD4+ T cells was not different in PBSC harvests compared to peripheral blood. The percentage of CD4+ or CD8+ T cells bearing activation markers CD69 and CD25, increased during the three consecutive days (day 9, 10 and 11) of the leucapheresis procedure (fig. 4).

Further in vitro activation of PBSC induced a marked increase in the expression of activation markers on CD8+ T cells. After 24 h of prior in vitro activation with

OKT3 and IL-2/OKT3, the percentage of CD8+ T cells also positive for early activation marker CD69 was increased (to mean 75% and 82% respectively, both $p<0.0005$ compared to the PBS control); whereas after 72 h the percentage of CD8+ T cells also expressing the late activation marker HLA DR was shown to be augmented (to mean 53% and 73% respectively, both $p<0.0005$). In the PBS control, no differences in activation markers was found after 0, 24 or 72 h. Although the percentage of CD8+ T cells in PBSC tended to rise during in vitro activation of PBSC with OKT3 and IL-2/OKT3, no significant difference was observed after 24 or 72 h activation as compared to 0 h. No difference in the total number of PBSC was found after 0, 24 or 72 h in the PBS control. No effect on the total number of PBSC was found after 24 and 72 h prior activation, compared to the PBS control. Also, no effect on lymphocyte and T cell subsets was observed after prior activation, as reflected in table 1.

Hematopoietic colony formation

Figure 5 shows the effect of prior PBSC activation on the ability of the hematopoietic stem cells to form hematopoietic colonies, measured as CFU-GM numbers. No effect of 24 h of prior activation was seen, when compared to the PBS control. The PBS control was not different after 24 or 72 h (mean 70 vs 67 CFU-GM, n=3, N.S.). Also after 72 h, no effect of prior PBSC activation with IL-2 or OKT3 was seen. However, after 72 h of IL-2/OKT3 activation, CFU-GM numbers were decreased (mean 39, n=3, $p<0.0005$). No negative effect of BIS-1 alone, or BIS-1 and GLC1M13 tumor cells, on CFU-GM numbers was observed after any of the prior PBSC activations (data not shown).

Discussion

In this study, we examined the possibility to use activation and retargeting of PBSC in vitro for purging of epithelial tumor cells from the PBSC isolate. As shown here, PBSC harvests from breast cancer patients appear to be intrinsically suitable for sustaining immunological purging procedures because they contain high levels of potential cytotoxic effector cells. This was also observed by Verma et al. (21). In our study, the capability of PBSC to lyse epithelial tumor cell was increased after in vitro activation with IL-2, OKT3 or IL-2/OKT3, and this was further augmented by the addition of the bispecific antibody BIS-1 (see fig. 1). Activation of PBSC was a prerequisite for effective BIS-1 mediated cell lysis, which is compatible with studies showing that T cells need prior activation in order to gain cytolytic potential (25 for review). By employing OKT3 activation of PBSC and subsequent BIS-1, within the four hours of the assay a tumor cell depletion of more than 3 logs was observed.

It can be argued that an even higher purging efficiency can be expected with this format in the clinical setting, for a number of reasons. PBSC harvests, without further purification except for erythrocyte lysis, were used for T cell activation and tumor cell kill. Selection by means of a density gradient was considered to be less desirable in view of the actual clinical situation. Thus, the “effector cells” consisted only for a minority of CD8+ T cells. Effector: target ratios in the clinical setting (i.e. the ratio of potential cytotoxic effector cells to tumor cells in tumor contaminated PBSC) are likely to be more than 1.10^2:1 (as used in the ^{51}Cr-release assay in this study) or 1.10^4:1 (log-depletion assay). This is generally the case since highly sensitive methods, including immunocytochemistry (32, 33) and reverse transcriptase-polymerase chain reaction

(RT-PCR) (34), are required to detect single tumor cells in 1.10^6 to 1.10^7 PBSC. Tumor cell lysis clearly increased with increasing E:T ratios, and therefore a high purging efficiency may be expected in the clinical setting.

The CFU-GM assay, which has predictive value for haematologic recovery after stem cell transplantation (35), was used as a functional evaluation of haematopoietic colony formation after the purging procedure in this study. CFU-GM numbers were not affected by PBSC treatment with OKT3, BIS-1 or even by tumor cell kill during the course of the cytotoxicity assay. In a number of studies, the use of antibodies for purging purposes was also found not to affect hematopoietic colony formation in vitro (20) or engraftment in patients (7-9,11). PBSC treatment with OKT3 was found to suppress hematopoietic colony formation in hematological malignancies (36), while normal control bone marrow was not affected (37). Furthermore, no adverse effect on hemapoiesis was seen in vivo when patients were treated i.v. with low dose OKT3 as used for induction of anti-tumor immunomodulation (38). The fact that we did not see a negative effect on PBSC of breast cancer patients of prior activation with OKT3 alone, is consistent with these findings. In vitro IL-2 incubation of breast cancer patient derived PBSC did not negatively affect hematopoietic colony formation in three studies (21, 22, 39). Our data confirm and extend these findings. In spite of this, the combination of OKT3 and IL-2 stimulation appeared to have a clear negative effect on hematopoietic colony formation in our study (fig. 5). In hematological malignancies, it was suggested that activated T cells could suppress hematopoietic colony formation (36). This might possibly explain our findings, as the degree of T cell activation after prior treatment with IL-2/OKT3 was indeed higher compared to the other treatments (for instance 73% CD8+ T cells also positive for HLA DR after 72 h IL-2/OKT3 activation, compared to 53% after OKT3 activation) in this study.

In search of purging methods both efficient in eliminating tumor cells and maintaining sufficient hemapoiesis, non-selective purging methods using chemotherapy failed to prove useful because of the negative effect on hematopoietic colony formation (5). As an alternative procedure, in vitro stem cell selection through enrichment of CD34 positive cells has been used, but tumor cells may not be completely eliminated this way (6). Antibody based purging methods, for instance with immunotoxins, proved efficient in eliminating tumor cells (3-4 log depletion, compatible with our results), but were shown to have varying effects on hematopoietic stem cells (19, 20). To find a universally expressed epitope in solid tumors is considered to be difficult, at least when compared to the situation in hematological malignancies (3). However, antibody based therapy using epitopes that are not universally expressed (15, 16, 18, 20) is obviously of little clinical significance. The method presented here may offer a good possibility for antibody based tumor elimination from hematopoietic stem cell harvests, as the EGP-2 transmembrane marker is not shed into the circulation, is frequently present and overexpressed in carcinoma cells, and is absent from bone marrow cells (26). The use of the patient material (PBSC) itself to eliminate tumor cells, is an additional asset of this method. Furthermore, highly sensitive methods for detection of tumor cells in peripheral blood and PBSC, i.e. immunocytochemistry and a quantitative RT-PCR based on EGP-2 expression, have been developed in our institute (34). This may allow us to evaluate our purging efficiency in clinically relevant patient samples, which may otherwise be potentially difficult.

It has been stated that an immunocompetent graft may provide anti-tumor activity, also concerning possible residual disease in the patient (3). Long-term follow-up analyses after CD34+ stem cell selection of PBSC grafts (which do not include the immunocompetent natural killer cells or T cells) may shed more light on the impact of immunocompetence of the graft. At this point, data on small numbers of patients are

available after a short follow-up, not allowing conclusions on disease free or overall survival as yet (40, 41). If indeed immunocompetence of the graft should play a role, the purging method with BIS-1 described here is likely of interest because immunocompetent cells remain in the graft. Both OKT3 and BIS-1 are used clinically, and the toxicity of OKT3 and BIS-1 is well known, in vitro (25-27), as well as in vivo (14, 27, 38). Autologous patient serum could replace NCS or HPS in this setting (own observation). Therefore, we are currently investigating the possibility to perform a clinical study including the use of OKT3 for T cell activation and retargeting by BIS-1 for purging epithelial tumor cells from PBSC.

The results of the present in vitro study indicate that specific purging of epithelial cancer cells by means of bispecific antibody BIS-1 is feasible and effective in vitro.

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Table 1:

The effect of processing on total PBSC numbers, lymphocytes and T cell subset fractions

	t=0	**t=72 h: PBS**	**OKT3**	**IL-2/OKT3**
Total no. PBSC *Per flask (10^6)*	10	10.8 (SD 3)	16.6 (SD 3)	15.5 (SD 2.2)
Lymphocytes *(% of total no. PBSC)*	47 % (SD 19)	53 % (SD 10)	60% (SD 12)	58% (SD 16)
CD 8 T cells *(% of lymphocytes)*	28 % (SD 10)	13 % (SD 8) (*)	24% (SD 12)	28% (SD 13)
CD4 T cells *(% of lymphocytes)*	50 % (SD 13)	40 % (SD 13)	37% (SD 13)	37% (SD 13)

Numbers prior to processing (t=0) and after 72 h prior PBSC activation with PBS (control), OKT3 or IL-2/OKT3 are reflected in this table (n=4). Numbers were not significantly different compared to t=0, except (*): $p<0.05$.

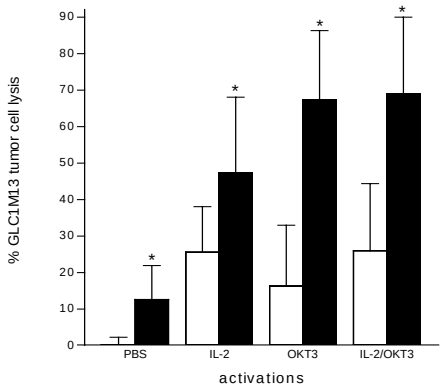

Figure 1: GLC1M13 cell lysis by PBSC with/without BIS-1

- On the Y-axis: % GLC1m13 tumor cell lysis; on the X-axis: activating agents. Open bars reflect activations without BIS-1; black bars represent activation with subsequent BIS-1.
- Shown is the % specific tumor cell lysis, determined in ^{51}Cr release assay (mean and SD, n=6) with E:T ratio 100:1, after 72 h prior activation. An * indicates significant difference compared to counterpart without BIS-1.

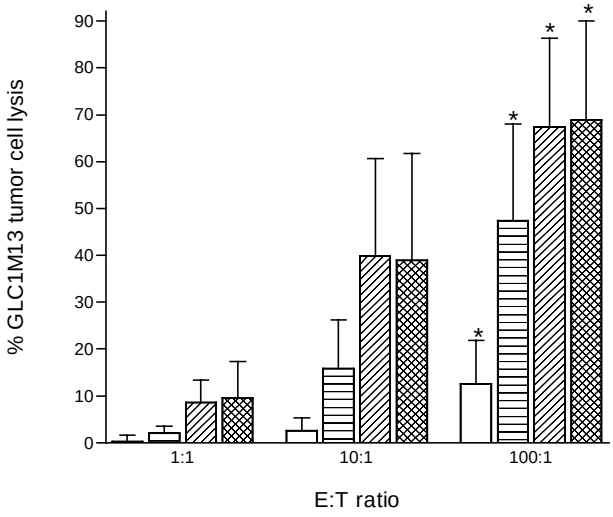

Figure 2: Effect of E:T ratio

- On the Y-axis: % GLC1M13 tumor cell lysis; on the X-axis: effector:target ratios 1:1, 10:1, 100:1 (=PBSC: tumor cell ratio).
- Shown is the % GLC1M13 cell lysis (mean, n=6) by PBSC + BIS-1 after 72h activation (PBS: open bar, IL-2: horizontal stripes, OKT3: oblique stripes, IL-2/OKT3: checkered bar). An * indicates significant difference compared to E:T ratios 1:1 and 10:1.

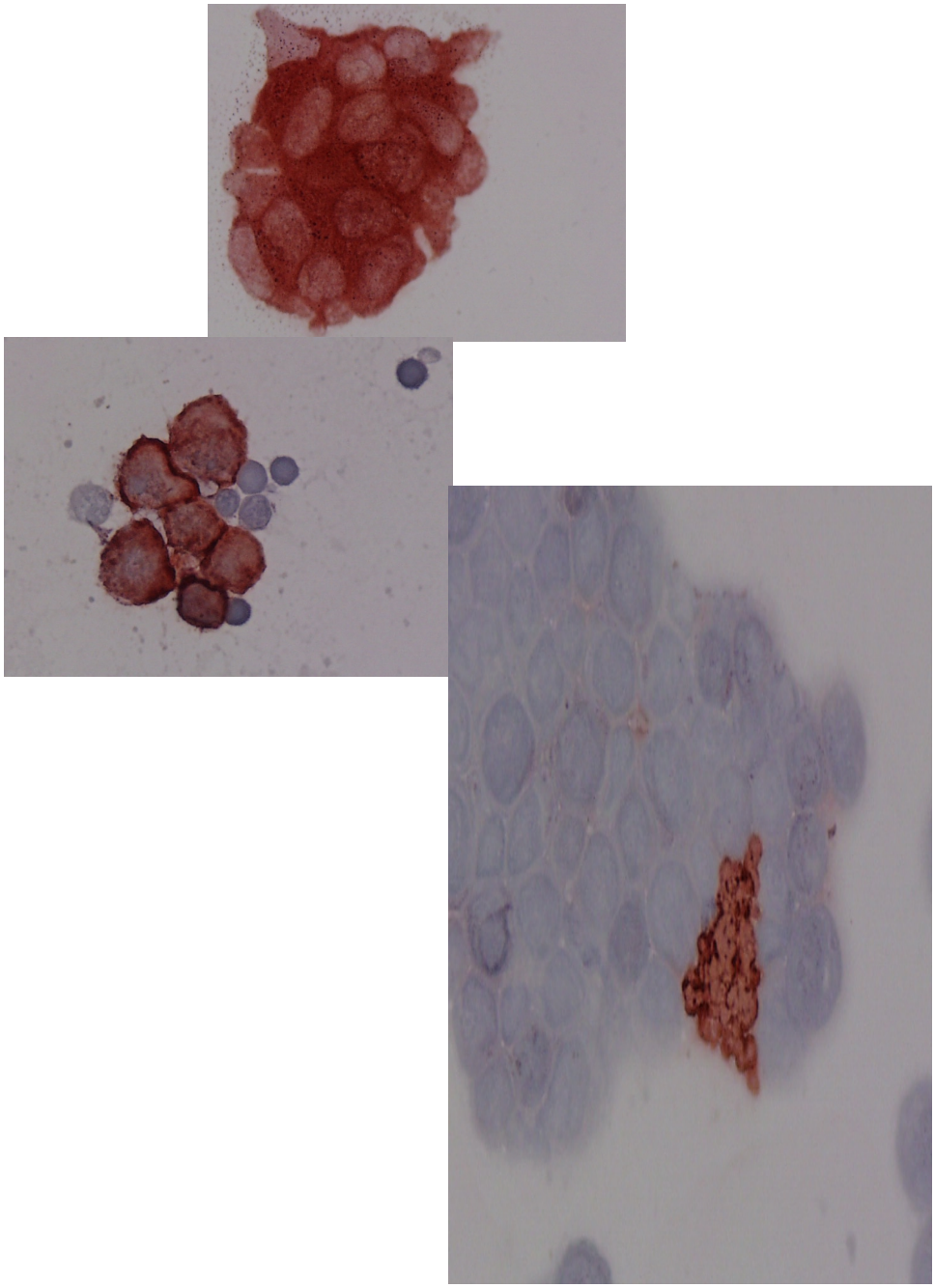

Figure 3: The effect of activated PBSC with BIS-1, on MCF-7

20x10 enlargement

- A: MCF-7 tumor cells without PBSC
- B: MCF-7 tumor cells with OKT3 activated PBSC, without BIS-1: viable appearance
- C: MCF-7 tumor cell with OKT3 activated PBSC, with BIS-1: non-viable appearance

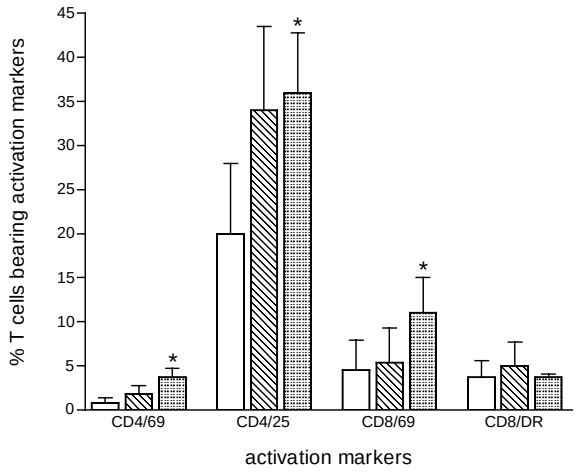

Figure 4: Activation markers on CD4+ and 8+ T cells in PBSC, during the three consecutive days of the leucapheresis procedure.

- On the Y-axis: % T cells with mentioned activation markers; on the X-axis: activation markers on CD4+ and CD8+ T cells.
- Shown is the % T cells in PBSC with marker (mean, n=3) on consecutive leucapheresis days: day 9 of the course: open bar, day 10 of the course: striped bar, day 11 of the course: checkered bar. An * indicates significant difference compared to first value on day 9.

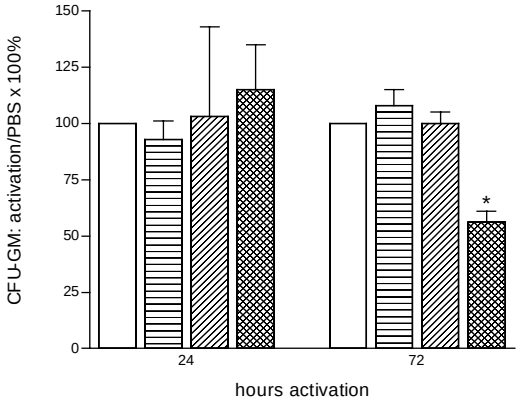

Figure 5: Hematopoietic colony formation after activation of PBSC.

- On the Y-axis: % hematopoietic colonies (CFU-GM) relative to PBS control (set at 100%); on the X-axis: 24 and 72 h activation.
- Shown is % hematopoietic colonies (mean and SD, n=3) after 24 or 72 h PBSC activation with PBS (open bar), IL-2 (horizontal stripes), OKT3 (oblique stripes) or IL-2/OKT3 (checkered bar). An * indicates a significant difference compared to PBS control.

List of abbreviations

PBSC:	peripheral blood stem cells
EGP-2:	epithelial glycoprotein-2
BIS-1:	bispecific antibody-1
IL-2:	interleukin-2
OKT3:	anti-CD3 antibody
CFU-GM:	granulocyte and macrophage-colony forming unit
PBS:	phosphate buffered saline solution
rhG-CSF:	recombinant human granulocyte-colony stimulating factor
FEC:	5-fluorouracil, epirubicin and cyclophosphamide
EDTA:	ethylene diamine tetra acetate
FCS:	fetal calf serum
NCS:	newborn calf serum
HPS:	human pooled serum
DMEM:	Dulbecco's Modified Eagle Medium
PE:	phycoerythrin
FITC:	fluorescein isothiocyanate
SCLC:	small cell lung carcinoma

An in vitro model for purging of epithelial tumor cells from cryopreserved ovarian tissue of women with impending fertility loss due to cancer treatment

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Submitted

Abstract

Introduction: Cancer treatment with chemotherapy and/or radiotherapy for the treatment of cancer can lead to impaired fertility in female patients. Cryopreservation and autografting of ovarian tissue is a promising new method for conserving their fertility. However, tumor cell contamination of the autograft may form a problem. Epithelial tumor cell lysis can be obtained with cytotoxic T cell retargeting through bispecific antibody BIS-1, which has a combined affinity for the T cell receptor and epithelial glycoprotein-2 (EGP-2). The aim of this study was to evaluate tumor cell kill (purging) and morphological follicle survival in an *in vitro* model with activated lymphocytes, BIS-1 and EGP-2 positive tumor cells, in the presence or absence of a thawed ovarian tissue. Methods: Thawed human ovarian tissue was carefully rendered into suspension by mechanical and enzymatical treatment. Cells of the MCF-7 breast cancer cell line and activated human lymphocytes were co-incubated for 4 h with/without 0.1 μg/mL BIS-1, in the presence or absence of ovarian suspension. After the purging procedure, MCF-7 cell kill was evaluated directly by means of fluorescent detection of remaining MCF-7 cells. Depletion of growing MCF-7 cells was assessed with an MTT assay, after 5 days. The morphology of ovarian tissue was evaluated immunohistochemically. Results: MCF-7 cell kill and depletion of cell growth increased with increasing ratio's of lymphocytes to MCF-7 cells, and the addition of BIS-1 further augmented this increase (to a maximum depletion of growing MCF-7 cells of 89%, SD 11%; $p<0.001$ compared to depletion without BIS-1). Follicles remained morphologically intact. Conclusions: These results show that purging of epithelial tumor cells from ovarian grafts with BIS-1 is possible in vitro. Morphologically, follicles remain intact after this procedure. This method may contribute to the safe replacement of ovarian tissue in female cancer survivors.

Introduction

In females, chemotherapy and/or radiotherapy for the treatment of cancer can cause a reduction of the follicle population within the ovaries, which can lead to a premature menopause (1-6). The cryopreservation of ovarian tissue obtained before cancer therapy is a promising new method for conserving the own fertility of these cancer patients prior to therapy (7). In animal studies the transplantation of frozen-thawed ovarian autografts has led to a resumption of endocrine function and the restoration of fertility (8-14). In one case-report, the successful re-transplantation of cryopreserved ovarian tissue into a previously oophorectomized woman with a non-malignant disease was described (15). In cancer patients however, there is concern that autografting of ovarian tissue can possibly reintroduce tumor cells (16).

Purging of minor quantities of tumor cells has been described in the hematopoietic stem cell transplantation setting (17, 18), but not for solid (tumor) tissue. The epithelial related membrane antigen (EGP-2) with a molecular weight of 38 kDa is known to be widely expressed on breast and ovarian carcinomas (19, 20). The bispecific antibody BIS-1, directed against EGP-2 on tumor cells and CD3 on T lymphocytes, creates functional cross-linking of T cells and tumor cells allowing the delivery of a tumor cell specific lethal hit inducing specific epithelial tumor cell kill in vitro and in vivo (21, 22). In peripheral blood stem cells, this approach resulted in a >3 log tumor cell depletion without affecting clonogenic potential of the hematopoietic stem cells (23). This study was conducted to evaluate whether solid ovarian tissue, rendered into suspension, can be purged in a similar way as hematopoietic stem cell material. Therefore, tumor cell kill and morphological follicle survival were studied in an *in vitro* model in which activated lymphocytes and BIS-1 were added

to the breast cancer cell line MCF-7, in the presence or absence of a suspension of human frozen-thawed ovarian tissue.

Materials and methods

In vitro model

Ovarian tissue

Freezing procedure

Human ovarian tissue, obtained with laparoscopy, was frozen from eligible patients since 1998. The freezing procedure of ovarian tissue for eventual transplantation purposes, was considered part of the regular patient care by the Medical Ethical Committee of our institution; the usage of ovarian tissue for the in vitro purging procedure (as described below), in case of the death of the patient prior to possible transplantation, was approved by the Medical Ethical Committee. All patients gave informed consent. The freezing- and thawing procedure was performed as described by Gosden's group (24). Briefly, after collection in sterile, buffered medium the ovary was cut into two parts under sterile conditions. One part was fixed in buffered formalin and embedded in paraffin after which sections for standard hematoxylin-eosin (HE) staining were cut; the other part was used for preparation of the ovarian cortex. Pieces of the cortex of approximately 3 x 3 mm, about 1 mm thickness were incubated for 30' in Leibovitz L15 medium (Life Technologies, Paisley, Scotland) containing 10% autologous patient serum and cryoprotecting agents (1M dimethyl sulfoxide (DMSO) and 0.1M sucrose). Thereafter, they were cooled to -140°C in a programmable freezer (Planer Kryo 10, series II; cooling with -2°C/min. up to -9°C, manual seeding at -9°C; cooling with 0.3°C up to -40°C followed by cooling with -10°C up to -140°C). Finally, the pieces were stored in liquid nitrogen.

Thawing and suspension procedure

Ovarian tissue was thawed in a water bath at 37°C for maximally 2 min, and washed in a diminishing sequence of DMSO in Leibovitz medium with 10% fetal calf serum (FCS, Life Technologies, Paisley, Scotland). First, the tissue was mechanically rendered into suspension with 27 G needles under sterile conditions. Then, enzymatical treatment was performed in medium containing 10 U/mL DNAse I (Sigma-Aldrich, Zwijndrecht, the Netherlands) and 300 U/mL collagenase IA (Sigma-Aldrich, Zwijndrecht, the Netherlands) for 2 h at 37°C. The obtained cell suspension was transferred into RPMI medium (Life Technologies, Paisley, Scotland) with 10% FCS. Next, into each well of a 96 well microtiter plate (Nunclon, Roskilde, Denmark), 100 µL of the cell suspension was distributed. After an overnight culture the wells were inspected with an inverted phase-contrast microscope. In 5 independent experiments, the wells in which microscopically intact follicles were observed were counted and the total suspension in these wells was used for purging experiments as described below.

Effector- and target cells; bispecific antibody

Target cells: fluorescent detection system

The MCF-7 breast cancer cell line was used as EGP-2 positive tumor model. Cells were plated in microtiter plates (Nunclon, Roskilde, Denmark) and cultured overnight for optimal adhesion. Cells were labeled with the fluorescent dye chloromethyl fluorescein diacetate (CMFDA, Molecular probes Europe BV, Leiden, the Netherlands) for 30 min. CMFDA toxicity was established by the percentage spontaneous cell detachment 24 h after labeling. MCF-7 cells (200, 500 or 1000 cells per well) were incubated with increasing concentrations of CMFDA (0.5, 1, 1.5, 5, 10, and 15 µM). Cell detection was adequate at 1.5 µM, without signs of toxicity, and therefore this concentration was used in subsequent experiments.

Effector cells

Lymphocytes were obtained from peripheral blood of healthy volunteers by Lymphoprep (Nycomed Pharma AS, Oslo, Norway) isolation, washed and incubated in vitro with anti-CD3 antibody (at 0.5 μg IgG /ml RPMI medium with 10% FCS) followed after 72 h by washing and a subsequent culture in the presence of recombinant human interleukin-2 (rh IL-2, Aldesleukin, Chiron, Amsterdam, The Netherlands) at 100 IU/ml RPMI medium with 10% FCS for 48 h. Thereafter, cells were washed, counted and resuspended in RPMI medium with 10% FCS. This sequence of adding activating agents was shown earlier to induce T lymphocyte activation (23).

BIS-1

The BIS-1-producing quadroma was produced in our department by fusion of the hybridomas RIV-9 and MOC-31, producing anti-CD3 (IgG3) and anti-EGP-2 (IgG1) antibodies respectively, according to De Lau et al (24). Preparation and purification was performed as described earlier (20). Briefly, BIS-1 was produced on large scale by means of a hollow fiber culture system (Endotronics, Minneapolis, MN). Purification of the hybrid antibodies (IgG3/IgG1) from parental-type antibodies, also produced by the quadroma, was performed by protein A column chromatography. BIS-1 $F(ab')_2$ was then produced by means of digestion by pepsin followed by G150 Sephadex gel filtration, and added to a 0.9% sodium chloride solution to obtain a final concentration of 0.2 mg/mL.

Purging procedure

Tumor cell kill, direct detection

Lymphocytes (effector cells) were co-incubated in a 96-well plate (Nunclon, Roskilde, Denmark), in the presence of 0.1 μg/mL BIS-1, at 37°C in a humidified, 5% CO_2-containing atmosphere, with 200, 500 and 1,000 MCF-7 tumor cells (target cells) labeled with CMFDA as described above. Ratio's of effector to target cells were 0:1, 100:1, 500:1 and 1,000:1, in a total volume of 200 μL RPMI medium with 10% FCS. After 4 h of co-incubation the amount of remaining tumor cells was counted directly by means of an inverted fluorescence microscope (Olympus IMT, Tokyo, Japan), at emmission wave length 516 nm, and extinction wave length 492 nm. Tumor cell kill was assessed in 5 independent experiments.

The effect of the presence of the ovarian cortex suspension on tumor cell kill efficiency was evaluated comparing tumor cell kill as described above to tumor cell kill in wells to which also 100 μL of ovarian cortex suspension (prepared as described above, after overnight culture) was added, in a total volume of 200 μL of RPMI medium with 10% FCS. The prior fluorescent labeling of tumor cells allowed assessment of tumor cell kill also in the presence of the ovarian cortex suspension. The effect of adding ovarian cortex suspension on tumor cell kill efficiency was assessed with ovarian tissue of 3 patients.

The percentage tumor cell kill was calculated as the amount of untreated tumor cells (control) minus the amount of remaining tumor cells after treatment, divided by control amount of tumor cells, times 100%.

Tumor cell kill, indirect detection

To evaluate longer-term effects of the purging procedure on the growing potential of tumor cells, lymphocytes (effector cells) were co-incubated in a 96-well plate (Nunclon, Roskilde, Denmark), in the presence of 0.1 μg/mL BIS-1, at 37°C in a

humidified, 5% CO_2-containing atmosphere, with 2,000 or 5,000 MCF7 tumor cells (target cells). Ratio's of effector to target cells were 0:1, 10:1, 20:1 or 50:1, in a total volume of 200 µL RPMI medium with 10% FCS (similar as described in experiment described above). After 4 h of co-incubation, the supernatant was removed. Cells were washed with fresh RPMI medium with 10% FCS and the supernatant was removed. Fresh medium (200 µL) was added and cells were cultured for 5 days (during which medium was refreshed one additional time) at 37°C in a humidified, 5% CO_2-containing atmosphere. To establish tumor cell survival/growth after 5 days, the cellular reduction of 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) by the mitochondrial dehydrogenase of viable cells to a blue formazan product was evaluated in a standard microculture tetrazolium assay (25). DMSO (100%, 200 µL) was used to dissolve the formazan chrystals, and the plate was read in an ELISA reader (Thermo Max, Molecular Devices, Sunnyvale, CA) at wavelength 490 nm. Comparisons were made with wells containing tumor cells and lymphocytes without BIS-1, tumor cells and BIS-1 without lymphocytes, or lymphocytes alone. In this experiment, no ovarian cortex suspension was added, as the described detection method does not allow discrimination between viable tumor cells or viable ovarian cells. Depletion of growing tumor cells was assessed in 4 independent experiments.

The percentage depletion of growing cells was calculated as the number of untreated tumor cells (control) minus the number of remaining tumor cells after treatment, divided by control number of tumor cells, times 100%. Data were corrected for background lymphocyte formazan production.

Follicle morphology

Morphology and viability of follicles were assessed before freezing or after freezing-thawing of tissue and before and after the purging procedure. Before and after freezing-thawing, pieces of tissue were fixed in buffered formalin, dehydrated through an alcohol series, and paraffin embedded. Before and after the purging procedure, ovarian cortex suspension was spotted on a glass slide. Slides of all material were stained with standard Giemsa staining as well as Periodic Acid Schiff method (PAS, 27), Papanicolau method (PAP, 27), and the MOC31 antibody, directed against EGP-2, for tumor cell presence. Evaluation criteria for morphology on paraffin sections were eosinophilia of ooplasm, clumping of chromatin and wrinkling of the oocyte nuclear membrane as signs of atresia (28).

Statistics

Tumor cell kill, assessed by means of direct (fluorescent) detection or indirect (MTT) detection, was analyzed by means of a two-sided Students' t-test for independent samples. Also the effect of adding ovarian cortex suspension on tumor cell kill was analyzed by means of this test. All analyses were performed with the statistical software program SPSS. A $p<0.05$ was considered statistically significant.

Results

Ovarian tissue

Ovarian tissue was used from 3 patients. The first two patients were 13 and 17 years old at the time of cryopreservation, and they suffered from acute lymphatic leukemia and an ovarian germ cell tumor of the contralateral ovary, respectively. From the third patient, aged 35 years, cryopreservation of ovarian tissue was performed after prophylactic ovariectomy because of a BRCA1 mutation; this patient gave consent for cryopreservation of ovarian tissue for the purging procedure described here. In the ovarian tissue of the latter patient, no sign of tumor contamination was present.

Purging efficiency

Tumor cell kill, direct detection

Figure 1A reflects the percentage kill of CMFDA labeled tumor cells after lymphocyte induced tumor cell kill in the presence or absence of BIS-1 for 4 h. In the absence of BIS-1, increased tumor cell kill is obtained with effector to target ratio's 500 and 1,000, compared to the control. This kill is further augmented by adding BIS-1, to a maximum at effector to target ratio 1,000 (kill 75.5 %, SD 10.5, $p=0.002$ compared to kill without BIS-1: 40%, SD 19). Tumor cell kill is similar as described earlier in hematopoietic stem cell harvests (23), with these effector to target ratio's.

The effect of the addition of ovarian tissue on tumor cell kill efficiency is reflected in figure 1B. Tumor cell kill in the presence of BIS-1 is increased with effector to target ratio's 500 and 1,000; there is no difference between tumor cell kill with or without added ovarian tissue.

Tumor cell kill, indirect detection

In figure 2, the percentage depletion of growing MCF-7 tumor cells is reflected, after a 4 h co-incubation with activated lymphocytes in the presence or absence of BIS-1 and subsequent culture for 5 days. Depletion of growing tumor cells is clearly increased after treatment with activated lymphocytes in the presence of BIS-1, compared to the absence BIS-1. A maximum tumor cell depletion of 89% (SD 11%, $p<0.001$ compared to depletion without BIS-1) at effector to target ratio 10 is seen.

Follicle morphology

Effect of freezing

No differences were observed between the tissue that had been passed through the freezing procedure and fresh tissue that was directly embedded in paraffin, when scored with the morphological criteria described in the materials and methods section.

Effect of thawing and suspension procedure

The effect of the mechanical and enzymatical suspension procedure after thawing, was evaluated in the ovarian cortex suspension after overnight culture, as described above. The number of wells (in the 96-wells plate) microscopically containing one or more follicles was mean 62, SD ±30. The suspensions in these wells that were not used for purging experiments, were paraffin embedded en scored with the morphological criteria described above. Intact follicles were detected in these suspensions, and a representative sample of frozen-thawed ovarian suspension is shown in figure 3.

Effect of purging

Morphological evaluation of the suspension including follicles, lymphocytes and tumor cells after the purging procedure, scored with the morphological criteria described above, revealed intact follicles remaining. A representative picture is shown in figure 4. No MOC31 positive tumor cells were detected after the purging procedure.

Discussion

Improvement in anticancer therapies has resulted in more long-term survivors. This has increased the awareness of long-term effects, such as gonadal failure (1-6). As there are only few possibilities to limit the toxic effect of chemotherapy and radiotherapy on ovarian function (29-31), there is a growing need to study the possibilities of ovarian protection and preservation.

Cryopreservation of ovarian tissue is a potential method to maintain fertility in females. In recent years, the procedure for freezing and thawing of ovarian tissue seems to be established (32). Primordial follicles, abundantly present in ovarian tissue of young women, were shown to survive the cryopreservation procedure relatively well (32). Restoration of fertility and endocrine function after the transplantation of cryopreserved ovarian tissue was shown in animals (8-14), and recently in a young woman with a non-malignant disease (15). In cancer patients however, the concern that autografting of ovarian tissue can possibly reintroduce tumor cells appears justified (16, 29). This issue would be resolved if primordial follicle isolation and subsequent in vitro maturation were possible. However, this technique is still in its infancy (33). Alternatively, one of the procedures for autografting of cryopreserved ovarian tissue involves reinsertion of a primordial follicle suspension in plasma clots. In the sheep model, this procedure already induced restored estrogenic activity and fertility (32). The preparation of a suspension of isolated follicles introduces the potential to clear, or purge the suspension from possible tumor cells. The same method for purging tumor cells as designed for peripheral blood stem cell harvests (23), may be applied to a suspension of follicle material. With this method, using bispecific antibody BIS-1 to retarget activated lymphocytes, specific tumor cell kill of > 3 logs was obtained while hematopoietic stem cell function remained intact. Since we demonstrated the

effectiveness of tumor cell purging in a breast cancer cell model, this was applied to the cryopreserved ovarian tissue setting also in this study. Moreover, this purging concept may very well be applicable to other tumor types such as B-cell lymphoma, which is sensitive to immunological treatment with monoclonal antibody rituximab (34), and for which a bispecific antibody was also developed in our institution (35).

The cryopreservation and thawing of ovarian tissue in this study was performed according to protocols described by the pioneering group of Gosden (24). The integrity of frozen-thawed follicles after enzymatic isolation, was confirmed by electron microscopy evaluation previously (24). Our results, showing morphologically intact follicles after thawing by light microscopy, are in line with this. For the purging procedure, subsequent to the thawing, a fluorescent detection system was developed to evaluate tumor cell depletion. As our ultimate aim is to culture the suspension of ovarian tissue after the purging procedure, the $Chromium^{51}$ release assay, commonly used to evaluate tumor cell depletion, was considered inadequate. With the fluorescent detection system, highly efficient tumor cell kill by activated lymphocytes in the presence of BIS-1 was demonstrated. No (adverse) effect of the presence of ovarian tissue on tumor cell kill was observed, and morphologically intact follicles were detected following the purging procedure.

This study supports the concept that solid tissue, rendered into suspension, can be purged in a similar way as hematopoietic stem cell material. In this in vitro setting to provide proof of principle, no adverse effect of the purging procedure on the morphological aspect of ovarian follicles was found. Future studies will address the important issue of the quantitative and functional survival of the follicles, according to studies performed by Hovatta et al. (28, 36, 37). Also, the potential of the above described method to clear tumor cells from a suspension of ovarian tissue with endogenous tumor cell infiltration will be investigated. As the enzymatic isolation of follicles most likely renders the endogenous tumor cells accessible for

lymphocyte cell kill, similar results are expected as in the described setting with the addition of exogenous tumor cells. To avoid potential aspecific lymphocyte activity directed against the ovarian tissue, the use of autologous patient lymphocytes will probably be preferred in a future patient related setting, although no such activity was observed in this study. In reference to the cell model chosen in this study, one might argue that restoring endocrine function and fertility is undesirable in breast cancer patients, because of possible hormonal growth stimulation of residual disease. However, there is no evidence so far that a pregnancy after breast cancer treatment increases the risk of poor prognosis (6, 38). With regards to the relevance of this study with a breast cancer cell model, it should be noted that a considerable number of breast cancer patients is diagnosed in childbearing years. In the Netherlands, this amounts to ± 1000 patients per annum: approximately 10% of women yearly diagnosed with breast cancer (39). Together with the trend towards postponed childbearing (40), preservation of fertility for these young cancer patients may become an issue of increasing importance.

Concluding, this study provides a first step into the direction of purging cryopreserved ovarian tissue from tumor cells. This would imply that patients with an increased risk of tumor cell contamination of the ovary, do not have to be excluded from gonadal cryopreservation beforehand. The safe replacement of ovarian tissue in female cancer survivors to restore their endocrine function and fertility, would be a major step forward in the improvement of the quality of life for these women.

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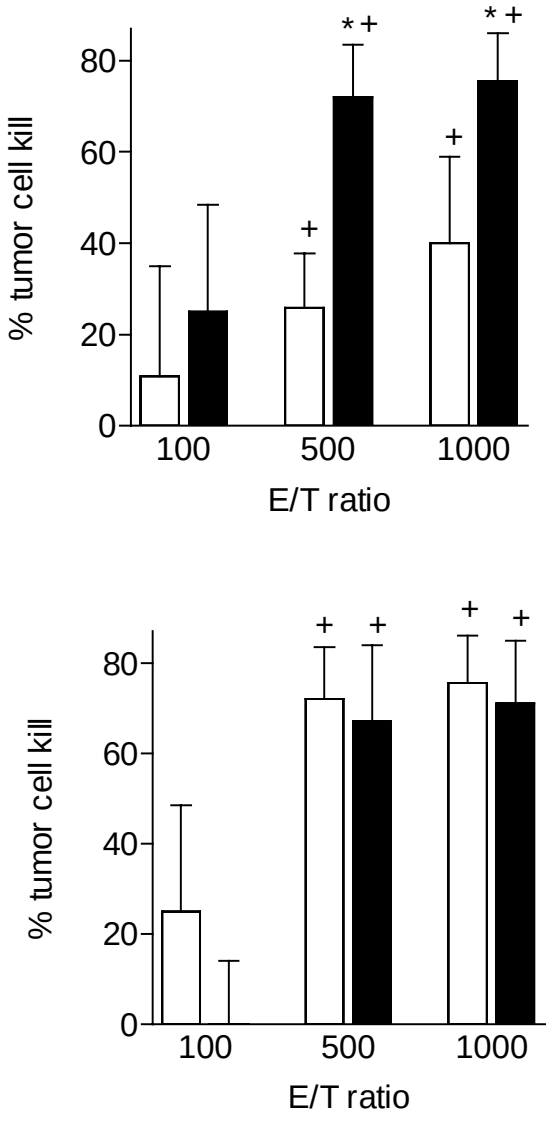

Figure 1: Direct assessment of tumor cell kill by fluorescent cell detection

X-axis: effector:target ratio's; Y-axis: percentage tumor cell kill of CMFDA labeled MCF7 tumor cells after 4 h co-incubation with activated lymphocytes, relative to the kill in untreated MCF7 cells. An (*) reflects a significant difference between the white and black bar; a (+) reflects a significant difference with the untreated control sample (kill 0%). **A**: effect of BIS-1. White bar: absence of BIS-1; black bar: presence of BIS-1. **B**: effect of ovarian cortex suspension in the presence of BIS-1. White bar: absence of ovarian cortex suspension; black bar: presence of ovarian cortex suspension.

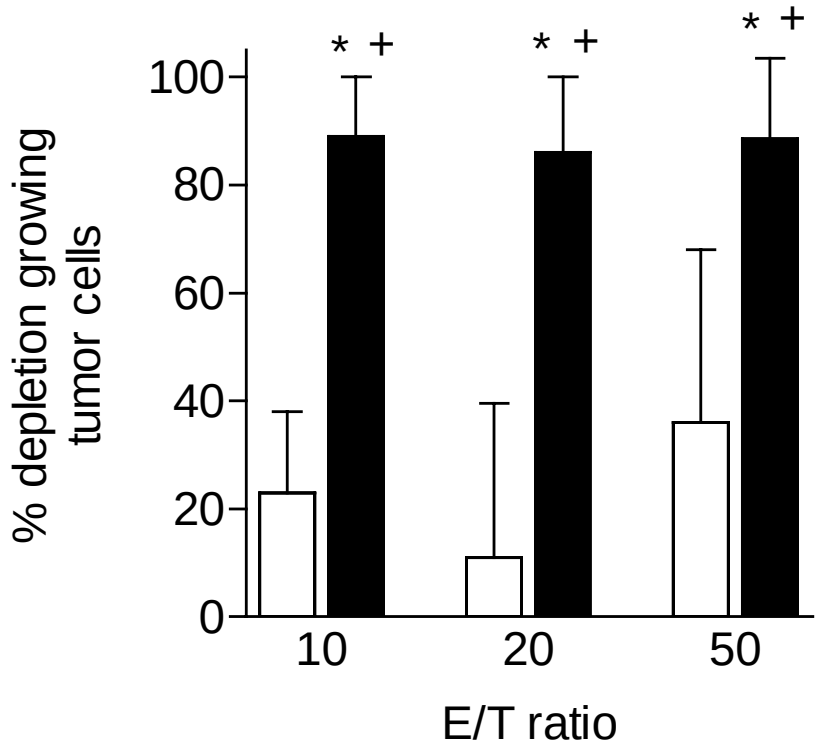

Figure 2: Indirect assessment of tumor cell kill by MTT assay

X-axis: effector:target ratio's; Y-axis: percentage depletion of growing MCF7 tumor cells after 4 h co-incubation with activated lymphocytes, and subsequent culture for 5 days, compared to tumor cell depletion in untreated MCF7 cells. White bar: absence of BIS-1, black bar: presence of BIS-1. An (*) reflects a significant difference between the white and black bar; a (+) reflects a significant difference with the untreated control sample (depletion 0%).

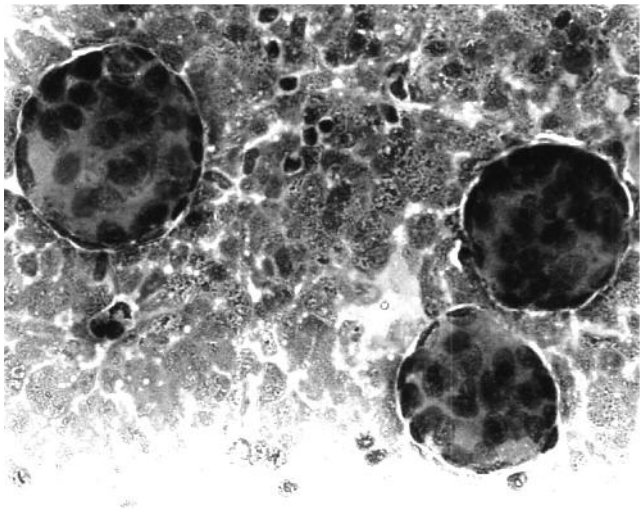

Figure 3: Effect of freezing-thawing procedure on follicle morphology

Shown is a representative piece of frozen-thawed ovarian tissue, at 40x10 magnification, with PAS staining. Three intact follicles are shown.

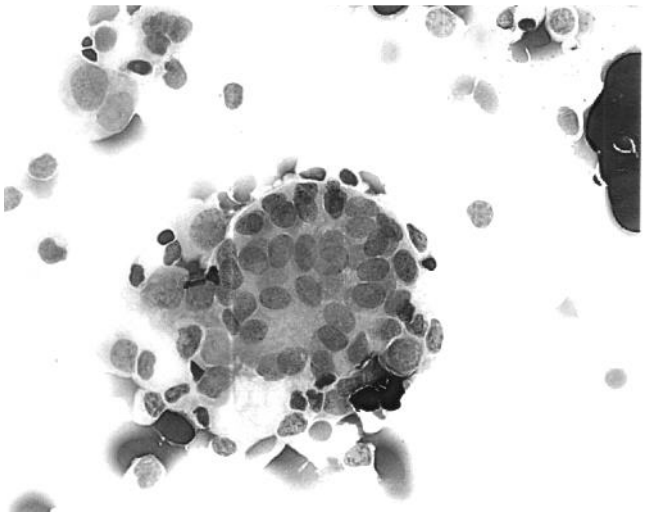

Figure 4: Effect of purging procedure on follicle morphology

Shown is a representative part of frozen-thawed ovarian tissue, after the purging procedure including activated lymphocytes and BIS-1, at 40x10 magnification with PAP staining. One intact follicle as well as lymphocytes are shown.

Prevention of febrile leucopenia after chemotherapy in high risk breast cancer patients: no difference between granulocyte-colony stimulating growth factor or ciprofloxacin + amphotericin B

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Summary

In a prospective randomized trial, 40 stage IV breast cancer patients undergoing intermediate high-dose chemotherapy (cyclophosphamide, 5-fluorouracil plus epirubicin or methotrexate), received either recombinant human G-CSF (rhG-CSF, group I) or ciprofloxacin and amphotericin B (CAB, group II) for prevention of febrile leucopenia (FL). In group I, seven of 18 patients developed FL (after 10/108 courses); in II: seven of 22 patients (7/98 courses) (p=N.S). Median hospitalization duration and costs were not different. RhG-CSF was 6.6 times more expensive per course than CAB. In conclusion, prophylactic CAB has similar efficacy as rhG-CSF in this setting, and is more cost-effective.

Introduction

Bacterial and fungal infection is a considerable cause of death in cancer patients, and chemotherapy related leucopenia, is associated with substantial febrile morbidity[1]. Prophylactic haematopoietic growth factors are used to reduce the incidence of FL, by shortening the duration of neutropenia[2,3]. Reducing the number of potential pathogens by means of prophylactic antibiotics and anti-mycotic agents, was also shown to lower the risk of febrile morbidity[4]. However, no prospective study to compare the efficacy of prophylactic haematopoietic growth factor or prophylactic antibiotics and anti-mycotics in preventing FL has been performed. The aim of this study was to evaluate the efficacy of prophylactic rhG-CSF or CAB, in patients with metastatic breast cancer treated with intermediate high-dose chemotherapy, in a prospective randomized clinical trial.

Patients and methods

Patients: chemotherapy naive patients ≤ 65 years of age, with metastatic breast cancer were treated with a chemotherapy scheme consisting of 3 courses of intravenous (IV) cyclophosphamide, epirubicin and 5-fluorouracil (5-FU) on day 1 (dosage 1,500, 80 and 1,500 or 1,000 mg/m^2 respectively). 5-FU dose-reduction was introduced for the latter 18 patients (see: discussion). These courses were followed by 3 courses of IV cyclophosphamide and 5-FU on day 1 (dosage 1,500 and 600 mg/m^2) and IV methotrexate on day 2 (1,500 mg/m^2). Informed consent was obtained according to local procedures. Courses were administered with an interval of 3 weeks.

Prophylactic treatment: prior to chemotherapy, patients were randomized to group I or II. Group I received rhG-CSF (lenograstim, Rhône-Poulenc Rorer Nederland BV, Amstelveen, The Netherlands) 263 μg subcutaneously once daily, on days 3 to 12. Group II received oral ciprofloxacin (ciproxin, Bayer Nederland BV, Mijdrecht, The Netherlands) 2 times 250 mg daily, and oral amphotericin B suspension (fungizone, Bristol-Myers Squibb BV, Woerden, The Netherlands) 100 mg/mL, 4 times 5 mL daily; both on days 3 to 17. Leucocyte counts were tested prior to the courses and once, between days 10 to 14 after start of the course.

Febrile leucopenia (FL): was defined as a leucocyte count <1.0.10^9/L (grade IV according to WHO toxicity scale[5]), combined with fever (temperature >38.5°C), and was followed by hospitalization and standard analyses of possible infectious foci. Treatment was started with IV broad spectrum antibiotics containing cefuroxim and aminoglycosides, and adjusted if necessary when a particular focus was found. Leucocyte counts were monitored daily. During hospitalization, rhG-CSF was continued in group I, whereas in

group II the ciprofloxacin was stopped, while the

amphotericin B was continued. Hospitalized patients from group II switched to the use of rhG-CSF during later courses according to protocol, based on prophylaxis guidelines after prior FL[6]. Patients were discharged when temperature had normalized (< 37.5°C) for at least 24 hours, and when leucocyte count was above $1.0.10^9$/L. No chemotherapy was administered during FL.

Cost analyses: *of* h*ospitalization*: were performed based on data by Vellenga et al.[7], regarding costs in our hospital for one day of treatment of FL on a regular oncology ward ($364) and additional costs per hospitalization (diagnostics etcetera, $590). Costs of antibiotic treatment during hospitalization were calculated for both groups. *Prophylaxis*: costs of CAB and rhG-CSF were based on whole sale prices.

Statistics: analyses were performed using the chi-square test with continuity correction according to Yates (incidence hospitalization for FL, grade IV leucopenia and FL), or the Mann-Whitney U-test (hospitalization duration and costs). Only p-values ≤0.05 were considered significant.

Results

A total of 40 patients were randomized. Patients' characteristics and metastatic sites are reflected in table 1. Group I consisted of 18 patients, receiving a total of 108 analyzed courses. Group II consisted of 22 patients receiving a total of 98 analyzed courses. Not included in the analyses were 23 courses from 7 patients from group II, who switched to rhG-CSF. Of these 7 patients, 3 patients stopped, due to disease progression or death of disease, after having received a total of 9 courses; therefore 11 more courses were not administered and not included in the analyses.

Hospitalization for FL: in group I, 7/18 patients were hospitalized after 10/108 courses for FL; in group II, 7/22 patients after 7/980 courses (p=N.S.). Prior to 5-FU dose-reduction, seven of nine patients (group I) and six of 13 (group II) suffered from FL (after 54 and 49 courses respectively, p=N.S.). After 5-FU dose-reduction for the last 18 patients studied, FL declined equally in both groups (I: 0/9 patients; II: 1/9). As shown in the Figure, FL occurred mainly after the first three courses. Median hospitalization duration was 6 days (range 5-9) for group I, and 7 days (range 5-10) for group II (p=N.S.). No course was delayed due to FL.

Grade IV leucopenia and FL: in group I, 22/108 courses were followed by grade IV leucopenia; in group II, 41 of 98 (20 vs. 42%, $p<0.0025$). In group I, grade IV leucopenia was followed by fever in 10/22 courses; in group II, seven of 41 (45 vs. 17%, $p<0.025$).

Cost analyses: *for hospitalization*: no difference was found between both groups regarding regular oncological care and additional costs (group I: median $2,774 per hospitalization, range $2,410-$3,866; group II: median $3,138, range $2,410-$4,230). Also costs of antibiotic treatment per hospitalization were comparable (group I: median

$332, range $40-$734; group II: median $439, range $108-$594). *Prophylaxis*: the costs of the prophylactic rhG-CSF were 6.6 times higher than CAB ($1,085 per course vs $164).

Discussion

In this study, the efficacy of prevention of FL by rhG-CSF or CAB was evaluated, in patients with metastatic breast cancer treated with intermediate high-dose chemotherapy, in a prospective randomized clinical trial. The results show no difference of the incidence of hospitalization due to FL in the two groups, whereas in the group receiving CAB, a larger number of patients appeared to be at risk for developing fever with a significantly higher incidence of grade IV leucopenia. Although the reduction of 5-FU dosage during the study clearly affected the overall incidence of FL (which was the objective, as the incidence of FL was considered unethically high), no difference in FL between groups was induced.

In a retrospective study, prophylaxis of FL with either rhG-CSF or ciprofloxacin was equally beneficial in patients with paclitaxel induced leucopenia compared to a historical control group[8]; however, no randomized prospective study addressing this issue was performed previously. From the study presented here, prophylactic CAB may be considered to be a reasonable alternative for rhG-CSF (standard in patients at high risk for FL[6]). The cost aspect adds to the attraction of this alternative. Placebo-controlled assessment of prophylactic antibiotic and anti-mycotic agents will be useful in future studies, preferably in patients with grade IV leucopenia (thus possibly reducing the risk of development of resistant organisms).

Concluding, prophylactic CAB appears to be an effective and attractive alternative for rhG-CSF in preventing febrile leucopenia in high risk patients, but future placebo-controlled studies will have to further support this.

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Table 1: **Patients' characteristics**

Characteristic		group I	group II
Age (years)	median	39	42
	range	28-50	29-51
Metastases	single	8	14
	multiple	10	8
Metastatic sites			
	supraclavicular LN	10	10
	bone marrow	4	5
	liver	4	4
	lungs	2	3
	pos. bone scan	9	7
	skin	1	3

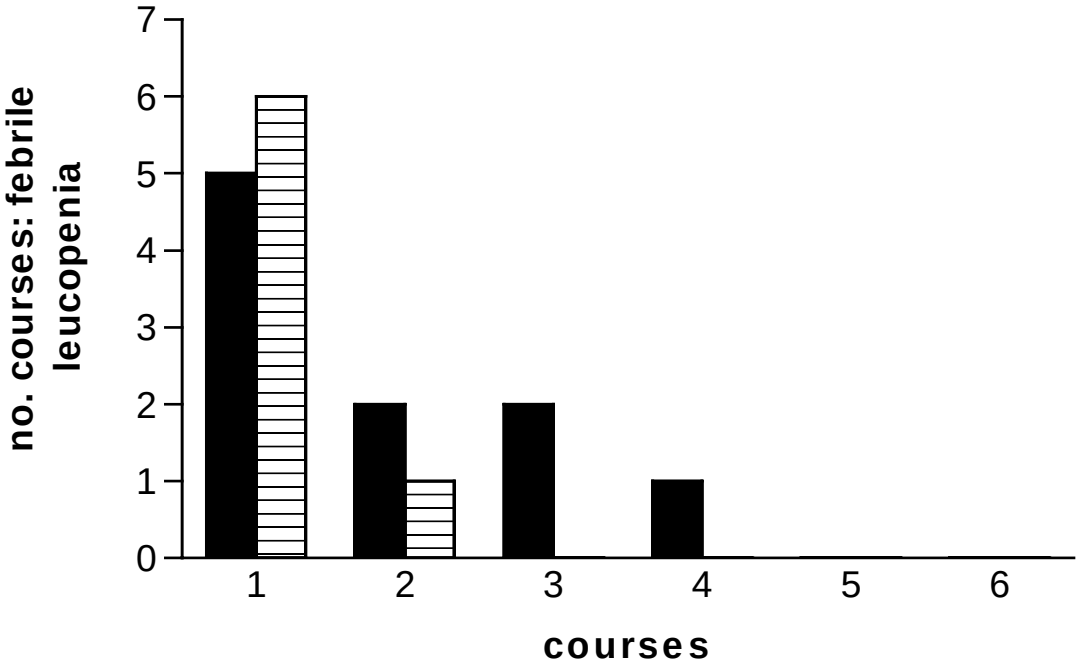

Figure: 1

Incidence of courses followed by febrile leucopenia.

X-axis: consecutive courses; Y-axis: number of courses followed by febrile leucopenia (black bar: group I, rhG-CSF; hatched bar: group II, CAB).

Telomere length in breast cancer patients before and after chemotherapy with or without stem cell transplantation

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Abstract

High-dose chemotherapy and peripheral blood stem cell transplantation (PBSCT) may accelerate telomere length loss in hematopoietic stem cells. As data including pre- and post-treatment samples are lacking, we studied leukocyte telomere length and telomerase activity before and after treatment in breast cancer patients randomised to receive 5 adjuvant courses FEC (5-FU, epirubicin and cyclophosphamide) (n=17), or 4x FEC followed by high-dose cyclophosphamide, thiotepa, carboplatin and autologous PBSCT (n=16). Haemoglobin, MCV, leukocyte- and platelet numbers were assessed prior to (t_0), 5 months after (t_1) and 9 months after chemotherapy (t_2); these parameters were decreased at t_1 and t_2 compared to t_0 (high-dose: all parameters; standard-dose: leukocytes and platelets), and all parameters were lower after high-dose than standard-dose treatment at t_1. Paired individual leukocyte samples of t_0 and t_1 showed telomere length change (determined by telomere restricted fragment (TRF) assay) ranging from +0.8 to –2.2 kb, with a decreased TRF length in 9 patients of both groups. Telomerase activity (determined by TRAP assay) was below detection limit in leukocyte samples of t_0 and t_1. Thus, standard- and high-dose chemotherapy negatively affect haematological reconstitution in this setting. In individual patients, telomere length can be remarkably changed following haematological proliferative stress after treatment.

Introduction

Human telomeres are regions at the chromosomal ends that play an important role in the structure and function of chromosomes. In normal somatic cells telomeres are shortened with every cell division, and when a critical size is reached, cells lose their proliferative potential (Harley *et al.*, 1990; Hastie *et al.*, 1990; Harley, 1997). Also in purified hematopoietic stem cells telomeric DNA appears to shorten with each cell division and thus with age (Vaziri *et al.*, 1994; Lansdorp, 1995). A number of studies have indicated a possible accelerated shortening of telomere length in hematopoietic stem cells, due to proliferative stress following peripheral blood stem cell transplantation (PBSCT) (Wynn *et al.*, 1998; Notaro *et al.*, 1997; Ball *et al.*, 1998; Shapiro *et al.*, 1996; Akiyama *et al.*, 1998; Lee *et al.*, 1999; Akiyama *et al.*, 2000). Although low levels of telomerase -a ribonucleoprotein that synthesises telomeric DNA- can be determined in CD34+ hematopoietic stem cells, this appears to be insufficient to compensate increased shortening of telomere length (Notaro *et al.*, 1997). Because of possible negative long-term effects of this shortening, including possible cytogenetic abnormalities (Ball *et al.*, 1998; Ohyashiki *et al.*, 1999), genomic instability preceding myelodysplastic syndromes (Ohyashiki *et al.*, 1994) and reduced response following hematopoietic stress (Rudolph *et al.*, 1999), this is clearly of clinical interest. However, most data so far are obtained from allogeneic transplantation settings (Wynn *et al.*, 1998; Notaro *et al.*, 1997; Ball *et al.*, 1998; Lee *et al.*, 1999; Akiyama *et al.*, 2000), in paediatric patients. Fewer data are available on the effect of autologous PBSCT (Shapiro *et al.*, 1996; Akiyama *et al.*, 1998; Lee *et al.*, 1999; Akiyama *et al.*, 2000), while paired data including pre-treatment samples are lacking.

As telomere length of nucleated blood cells was shown to be widely variable between age-matched individuals (Wynn *et al.*, 1998; Notaro *et al.*, 1997; Ball *et al.*, 1998; Akiyama *et al.*, 1998; Lee *et al.*, 1999), prospective paired data are essential for determining the impact of autologous PBSCT on this possible ageing process. Therefore, we prospectively studied leukocyte telomere length and telomerase activity in a group of high-risk breast cancer patients randomised to receive either adjuvant standard-dose chemotherapy, or adjuvant high-dose chemotherapy and PBSCT (De Vries *et al.*, 1996). Paired samples before and after these treatments were compared, allowing assessment of the impact of standard and high-dose chemotherapy on telomere length and telomerase activity.

Patients and methods

Patients

Patients included in this study participated in a national randomised adjuvant breast carcinoma study (De Vries *et al.*, 1996). Chemotherapy naive breast cancer patients with four or more tumour-involved axillary lymph nodes (stage II and III), ≤ 55 years of age with negative chest X-ray, liver ultrasound and bone scan, were randomised to receive 5 courses of standard-dose chemotherapy followed by radiotherapy, or 4 courses of the same combination chemotherapy followed by high-dose chemotherapy, PBSCT and radiotherapy. These groups will be referred to as the standard-dose group, and the high-dose group, respectively. The combination chemotherapy consisted of 5-fluorouracil (500 mg m^{-2}), epirubicin (90 mg m^{-2}) and cyclophosphamide (500 mg m^{-2}), administered intravenously once every 3 weeks. For the high-dose group, PBSC were mobilised following the third or last course of FEC with daily subcutaneous recombinant human granulocyte-colony stimulating growth factor (rhG-CSF, 263 μg), from day 2 of the course. Leucapheresis was performed from day 9 of this course, until $\geq 5.10^6$ CD34+ cells kg^{-1} body weight (as determined by flow cytometric analysis with the fluorescein isothiocyanate-labelled anti-CD34 antibody directed against the HPCA-2 epitope on CD34+ cells, Becton Dickinson, Leiden, the Netherlands) were obtained. High-dose chemotherapy consisted of cyclophosphamide (1500 mg m^{-2}), thiotepa (120 mg m^{-2}) and carboplatin (400 mg m^{-2}) on days -6, -5, -4 and -3, followed by reinfusion of PBSC on day 0. After reinfusion, daily subcutaneous rhG-CSF was administered until the leukocyte count exceeded 3.10^9 l^{-1}. Locoregional radiotherapy (50 Gy in 25 fractions) was administered after completion of the chemotherapy scheme with sufficient bone marrow recovery (defined as platelets $>100.10^9$ l^{-1}). Oral tamoxifen 40 mg daily was administered after platelet recovery for two years, in both groups. The study, and

the collection of blood samples as described, was approved by the Medical Ethical Committee of the University Hospital Groningen. All patients gave informed consent.

Sampling times

Blood samples were collected from all consecutive patients randomised in this study from May 1997 until January 1999. Sampling times were: t_0: directly prior to start of chemotherapy; t_1: 5 months after completion of chemotherapy; t_2: 9 months after completion of chemotherapy.

Telomere length was measured in samples from t_0 and t_1. In a number of these samples it was also possible to measure telomerase activity. Haematological examinations, e.g. haemoglobin, mean corpuscular volume (MCV), leukocytes, and platelets were performed at t_0, t_1 as well as t_2. Haematological parameters were considered normal with haemoglobin ≥ 7.45 mmol l^{-1}, MCV 80- 96 fL, leukocytes ≥ $4.0.10^9$ l^{-1} and platelets ≥ 150.10^9 l^{-1} (Barbui *et al.*, 1996).

Analysis of telomere length

In blood samples from t_0 and t_1, lysis of erythrocytes was performed with an ammonium chloride solution (155 mM NH_4Cl, 10 mM potassium hydrogen carbonate, 0.1 mM sodium ethylene diamine tetra acetate, EDTA). The remaining nucleated cell fraction was then washed in phosphate buffered saline (PBS) solution (0.14 M NaCl, 2.7 mM KCl, 6.4 mM $Na_2HPO_4.2H_2O$, 1.5 mM KH_2PO_4, pH 7.4). After centrifugation (150 g, for 10 min) the supernatant was decanted and 1.10^6 cells of the pellet of nucleated cells were transferred onto a slide for assessment of the leukocyte differentiation. The remaining pellet of nucleated cells was stored at -80°C . This erythrocyte lysis procedure was performed in accordance with Wynn *et al.* (1998).

In the nucleated leukocyte cell fraction, mean telomere length was determined by the terminal restriction fragment (TRF) assay according to Harley *et al.* (1990), with minor modifications. DNA was isolated using the salt extraction method as described by Miller *et al.* (1988). 5 µg DNA was digested overnight at 37°C using 20 U *Rsa*I and 20 U *Hin*fI (Roche Diagnostics, Almere, The Netherlands). Digested DNA was electrophorised in a 0.6% agarose gel in 0.5x Tris-borate EDTA buffer overnight at 50 V. DNA was depurinated with 0.25 M HCl, denatured in 0.5 M NaOH and 1.5 M NaCl and neutralised in 0.5 M Tris/HCl (pH=7.5) and 1.5 M NaCl, after which the DNA was transferred to a positively charged nylon membrane (Roche Diagnostics, Almere, The Netherlands) using 10x SSC overnight and dried for 2 hours at 80°C. Prehybridisation, hybridisation with 5 ng ml^{-1} probe and washing were performed according to TeloQuant assay (PharMingen, San Diego). Preincubation, incubation with 1:5000 alkaline phosphatase conjugated streptavidine and washing were performed according to biotin luminescence detection kit instructions (Roche Diagnostics, Almere, The Netherlands). Telomeric smears were visualised by incubation of the membrane with the chemiluminescence substrate CPD-Star (1:100), according to the supplied instructions (Tropix, Westburg, Leusden, the Netherlands) and exposure to a film. Films were analysed using a scanner and Diversity One PDI computer software (Pharmacia Biotech, Roosendaal, the Netherlands). The mean TRF lengths were calculated with the formula: mean TRF length= $\sum(OD_i)/\sum(OD_i/L_i)$, in which OD_i is the density output and L_i is the length of the DNA at row i (normally a Gaussian curve was obtained) (Wynn *et al.*, 1998). For standardisation, DNA isolated from leukocytes (one sample) of one healthy donor was included on all gels. The mean TRF length of the leukocytes of the donor was 7.3 kb. TRF lengths from patient samples were normalised to the TRF length of the healthy donor sample, which was set at 7.3 kb for each gel analysed. Paired patient samples were always analysed on the same gel.

The intra-assay variance coefficient in this study was determined to be 1.4% (95% CI) after analysis of 10 aliquots of the control healthy donor sample on one gel, resulting in a mean measurement variance for each sample of ± 100 bp. Therefore, samples of individual patients with a difference in TRF value < 0.2 kb were considered equal. As control, the plasmid pTSK8 (linearized with *Kpn*I; a kind gift from Dr. Royle, Leicester, U.K.) was used, which contains approximately 200 base pairs (bp) of TTAGGG repeats (Royle *et al.*, 1992). TRF length change (Δ TRF length) was defined as the TRF value at t_1 minus the value at t_0.

Telomerase activity (the TRAP assay)

After obtaining the nucleated leukocyte cell fraction as described above, 1.10^6 leukocytes per telomerase activity assay were lysed in 100 µL TRAP lysis buffer (0.5% CHAPS; 10 mM Tris/HCl (pH 7.5); 1 mM $MgCl_2$; 1 mM EGTA; 10% glycerol; 5 mM ß-mercaptoethanol; 0.1 mM PMSF) and incubated on ice for 25 min. After centrifugation at 15,000 g for 20 min at 4°C, the supernatant was quickly frozen in liquid nitrogen and stored at - 80°C until further processing.

The TRAP assay was performed as previously described (Wisman et al., 1998). In short, telomerase activity levels in leukocytes were determined with a fluorescence-based telomeric repeat amplification protocol assay using GLC_4 cells (Zijlstra et al., 1987) as standard in each assay. Peaks representing telomerase activity in GLC_4 cell equivalents were summed, then relatively expressed to telomerase activity of 100 GLC_4 cell equivalents (set at 100%) and normalised to the signal of modified-internal telomerase assay standard (M-ITAS). For the samples (1.10^5 cells and 1.10^4 cells) the peaks representing telomerase activity were also summed and normalised to the signal of M-ITAS, thereafter the relative telomerase activity of the leukocytes was correlated to GLC_4 cell number (relative quantification comparable to 10 GLC_4 cell equivalents = 10 U).

Statistics

Mean TRF length and telomerase activity in blood samples from in individual patients were compared from t_0 and t_1, and statistically analysed with the Wilcoxon signed ranks test for paired samples. Comparisons of haematological parameters and leukocyte differentiations between the standard- and high-dose groups, were performed with the Student's t-test for independent samples, and comparisons between time points in both groups were performed with the t-test for paired samples. Correlations between TRF data, telomerase activity, numbers of CD34+ cells and haematological examinations were examined with the Pearson correlation test. All analyses were performed using the statistical analysis program SPSS. A $p<0.05$ was considered statistically significant.

Results

Patients

The standard-dose group consisted of 17 patients, and the high-dose group of 16. Mean age at the start of treatment was 44.0 years (range 29-54 years) and 44.6 years (range 37-54 years) in these groups respectively (N.S.). Mean period between blood samples of t_0 and t_1 was 32 weeks in the standard-dose group, and 37 weeks in the high-dose group (N.S.).

Haematological parameters

The analysis of haematological parameters is reflected in figure 1.

Leukocytes: Compared to the standard-dose group, leukocyte counts were lower in the high-dose group at t_1 (standard-dose: mean at t_1 $5.9.10^9$ l^{-1}, high-dose: mean $4.1.10^9$ l^{-1}, $p = 0.008$; leukocytes $< 4.0.10^9$ l^{-1} in 2/17 versus 7/16 patients respectively). At t_2 this difference was not observed.

Compared to t_0, a decreased leukocyte count was shown at t_1 in paired samples after both standard- and high-dose treatment ($p<0.001$); this difference remained present at t_2 in both groups (fig.1A).

Platelets: Compared to the standard-dose group, platelet counts were lower in the high-dose group at t_1 (standard-dose: mean at t_1 220.10^9 l^{-1}, high-dose: mean 137.10^9 l^{-1}, $p < 0.001$; platelets $< 150.10^9$ l^{-1} in 0/17 versus 11/16 patients respectively). At t_2 this difference was not observed.

Compared to t_0, a decreased leukocyte count was shown at t_1 in paired samples after both standard- and high-dose treatment ($p < 0.001$); this difference remained present at t_2 in both groups (fig. 1B).

Haemoglobin: In the standard-dose group, haemoglobin values were not different at t_0, t_1 and t_2. Compared to the standard-dose group, haemoglobin was lower in the high-dose group at t_1 (standard-dose: mean at t_1 8.0 mmol l^{-1}, high-dose: mean 6.7 mmol l^{-1}, $p < 0.001$; haemoglobin < 7.45 mmol l^{-1} in 2/17 versus 14/16 patients respectively) as well as at t_2 ($p=0.003$).

Compared to t_0, a decreased haemoglobin was observed at t_1 in paired samples after high-dose treatment ($p<0.001$); this difference remained present at t_2 (fig. 1C).

MCV: In the standard-dose group, MCV values were not different at t_0, t_1 and t_2. Compared to the standard-dose group, MCV values were increased in the high-dose group at t_1 (standard-dose: mean at t_1 90.9 fL, high-dose: mean 97.9 fL, $p < 0.001$; MCV > 96 fL in 2/17 versus 10/16 patients respectively), but not at t_2.

Compared to t_0, an increased MCV value was observed at t_1 in paired samples after high-dose treatment ($p<0.001$); this difference remained present at t_2 (fig 1D).

CD34+ cell number and haematological parameters

At t_1 in the high-dose group, the number of reinfused CD34+ cells (times 10^6 per kg body weight) correlated positively with the number of leukocytes ($r= 0.63$; $p =0.009$) and platelets ($r= 0.77$; $p <0.001$), and negatively with MCV ($r= -0.6$, $p =0.014$). No relation between haemoglobin and CD34+ cells was found. At t_2, no correlation between CD34+ cells and haematological parameters was observed.

Telomere length and telomerase activity

TRF length (mean of all patients at t_0 8.1 kb, SD 1.4) was in the same range as previously reported in cross-sectional studies (Wynn *et al.*, 1998; Ball *et al.*, 1998; Akiyama *et al.*, 1998; Lee *et al.*, 1999). As shown in figure 2, TRF length decreased

in 9 patients of each group when t_0 and t_1 samples were compared, and 4 patients from the standard- and 5 patients from the high-dose group showed a TRF length increase (mean Δ TRF length of both groups: -0.2 kb, SD 0.6; range Δ TRF length: standard-dose group: +0.4 to -2.2 kb; high-dose group +0.8 to -1.1 kb). Paired analysis of t_0 and t_1 samples showed overall no effect on TRF length of either treatment arm (standard-dose group: p=0.069; high-dose group: p=0.67) or of treatment in general (both groups together: p=0.148). A representative blot is shown in figure 3. No difference in leukocyte differentiation was found when t_0 and t_1 samples were compared of both groups, and no difference between the groups was observed at t_0 or t_1.

In the high-dose group, no correlation between reinfused CD34+ cells and actual TRF length at t_1, or Δ TRF length could be observed (fig 4).

Also the relation between haematological parameters haemoglobin, MCV, leukocyte- and platelet counts at t_1 and t_2 and TRF length, or Δ TRF length was evaluated. No correlation between these haematological parameters and (Δ) TRF length could be observed.

In 9 patients from each group, paired leukocyte sample size also allowed measurement of telomerase activity at t_0 and t_1. This included the samples with maximum TRF length increase or decrease of both groups. Telomerase activity in all of these patient samples was below the reliable detection limit of 10 U (equivalent to 10 GLC4 cells) per 1.10^5 leukocytes (Wisman *et al.*, 1998), in both groups at t_0 and t_1. This activity level is comparable with telomerase activity found in leukocytes from healthy controls (Wolthers *et al.*, 1999). Therefore, no strong up-regulation of telomerase activity was observed, also not in patients with increased TRF lengths after treatment.

Discussion

The perception that hematopoietic proliferative stress may accelerate the ageing of hematopoietic stem cells has gained interest, in view of the wide spread use of hematopoietic stem cell transplantations for various clinical conditions. Evidence for accelerated telomere shortening after hematopoietic stem cell transplantations was found in a number of studies (Wynn *et al.*, 1998; Notaro *et al.*, 1997; Ball *et al.*, 1998; Shapiro *et al.*, 1996; Akiyama *et al.*, 1998; Lee *et al.*, 1999; Akiyama *et al.*, 2000). Most data were derived from paediatric patients with haematological malignancies, and frequently mean TRF lengths after therapy were compared to mean TRF lengths of age-matched controls. However, mean TRF length of nucleated blood cells has been shown to be widely variable between these controls (Wynn *et al.*, 1998; Notaro *et al.*, 1997; Ball *et al.*, 1998; Akiyama *et al.*, 1998; Lee *et al.*, 1999). Additionally, samples were drawn at a wide range of time after PBSCT, ranging from 1.6 months (Lee *et al.*, 1999) to over 10 years (Akiyama *et al.*, 1998; Wynn *et al.*, 1999). Finally, as TRF dynamics were shown to be different in the various stages of life (Zeichner *et al.*, 1999), predictive value for the adult setting may not automatically be assumed from these paediatric data. Therefore, we studied mean leukocyte TRF length in paired samples before and after treatment, in a group of high-risk breast cancer patients randomised to receive either adjuvant standard-dose chemotherapy, or adjuvant high-dose chemotherapy and PBSCT. These treatment modalities are frequently used for breast cancer (Antman *et al.*, 1997), and their induction of hematopoietic stress and possible consequent effect on individual TRF length could be assessed. TRF length measurement in this study was performed based on the commonly used procedure by Harley *et al.* (1990), and care was taken to standardise measurements. In analogy to the pioneering study by

Wynn *et al*. (1998), we chose unselected leukocytes to measure TRF length and telomerase activity in. In recent studies, it has been suggested that lymphocytes may have a larger TRF length than neutrophils (Wynn *et al*., 1999; Robertson *et al*., 2000). Although TRF length of T lymphocytes and neutrophils was shown to be equally affected by stem cell transplantation (Wynn *et al*., 1999), in case of a change in the relative proportions of these cells, it might be slightly more difficult to draw conclusions from overall leukocyte TRF length. However, in this study no difference in the leukocyte differentiations was found in either group before or after treatment, and TRF length in our leukocyte samples is therefore unlikely to be affected by such a difference. Furthermore, variable differences of TRF length of neutrophils and T lymphocytes have been reported, ranging from approximately 1 kb (Wynn *et al*., 1999) to none (Martens et al., 2000). In light of these data, we consider leukocytes, in line with Wynn *et al*. (1998), sufficient for the purpose of this study.

Hematopoietic proliferative stress to achieve haematological reconstitution after treatment, was analysed by means of haematological parameters in peripheral blood, until 9 months after treatment. A clear negative effect on all haematological parameters was seen after high-dose treatment, and 9 months later still no recovery was made to the pre-treatment level. Even after standard-dose treatment, leukocyte- and platelet counts were significantly affected for at least 9 months. A long-term impact of PBSCT on haematological reconstitution was observed in haematological malignancies (Barbui *et al*., 1996). Our data appear to support this in the solid tumour setting also, but data from longer follow-up periods are needed to confirm this. In line with previous studies (Faucher *et al*., 1996; Bernstein *et al*., 1998), we found that the number of reinfused CD34+ cells correlated with leukocyte- and platelet numbers as well as MCV values, shortly after high-dose treatment.

Following the evident hematopoietic stress induced by both treatment arms (and PBSCT in particular), TRF length was clearly changed in individual patients. The majority of patients (n=9 in both arms) showed a TRF length decrease at t_1, but also remarkable TRF length increases were observed; no significant decrease due to either treatment was found in paired samples. The high-dose treatment scheme used in this study is classically combined with stem cell support in view of its profound myolotoxicity, causing prolonged life threatening marrow aplasia (Ayash *et al.*, 1993; Antman *et al.*, 1994). It is possible that in individual patients the lack of TRF length decrease due to treatment may be interpreted as a sign of insufficient treatment toxicity, as stem cells remaining in the patient after high-dose treatment will have an impact on the requirements to divide for haematopoietic reconstitution. In line with the presumed ablative nature of the treatment regimen in our study however, its profound impact on hematological parameters is clear. The maximum myelosuppression at t_1 and the (partial) hematological recovery at t_2, indicate hematopoietic proliferative stress at the time-point at which TRF length was measured (at t_1). Full recovery of hematological parameters after this high-dose treatment may actually take years (Nieboer *et al.*, 2000), and the impact of this lengthy process on TRF length changes at later time-points than t_1 is currently being studied.

The detection of a distinct increase of TRF length in some patients was surprising. We hypothesised that up-regulation of telomerase activity in response to replicative stress might be responsible for this, in agreement with in vitro studies with purified CD34+ cells (Engelhardt *et al.*, 1997; Yui *et al.*, 1999). However, in our samples telomerase activity remained undetectable after treatment. In drawing conclusions from this, it should be considered that telomerase activity is a much more dynamic parameter than TRF length. Possibly, telomerase activity changes took place at other time-points than were measured in this study. Furthermore, in

contrast to the comparable TRF length of leukocytes and CD34+ cells (Kronenwett *et al.*, 1996), telomerase activity in purified CD34+ cells is likely higher than in terminally differentiated cells such as leukocytes (Engelhardt *et al.*, 1997).

CD34+ cell numbers in our study were not related to (Δ) TRF length. Previously, it was assumed that if small numbers of CD34+ cells are reinfused, these cells may have to undergo more cell divisions than larger numbers, for a similar net hematopoietic effect (Notaro *et al.*, 1997). However, no relationship was found between the degree of TRF length shortening and the number of reinfused CD34+ cells in recent studies (Lee *et al.*, 1999; Wynn *et al.*, 1999) and our data support this. Possibly, in vitro culturing of CD34+ cells may provide more insight into the balance between cell proliferation and the ability to upregulate telomerase activity in individuals, leading to (change of) telomere length in vivo. Disturbances in this balance may be related to haematological malignancies (Engelhardt *et al.*, 2000). In this respect, the ability to measure TRF length in individual chromosomes or cells by means of flow cytometry (Rufer *et al.*, 1998) or in situ hybridisation (Martens *et al.*, 2000) may be of interest. It remains conceivable that a rapid TRF decrease, predisposes for long-term effects such as secondary malignancies in individual patients. This has to be evaluated after a longer period of follow-up.

In conclusion, in this study we found that standard- and high-dose chemotherapy (in particular) negatively affect haematological reconstitution. Leukocyte TRF length was remarkably changed in individual patients after treatment, showing both decrease (in the majority of patients), as well as increase. Therefore, although no accelerated telomere loss was observed in general, TRF length was clearly affected following proliferative stress in this setting.

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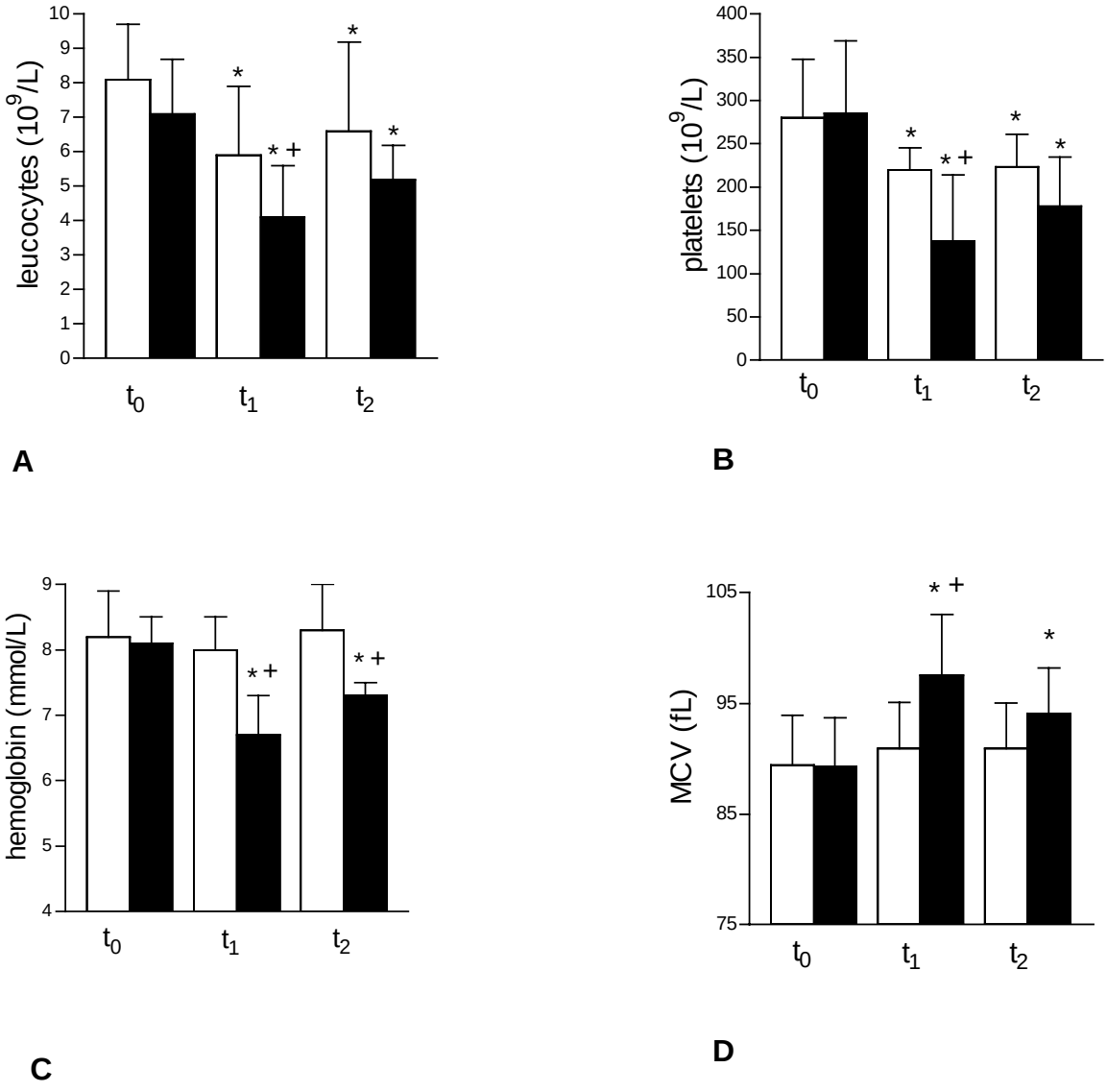
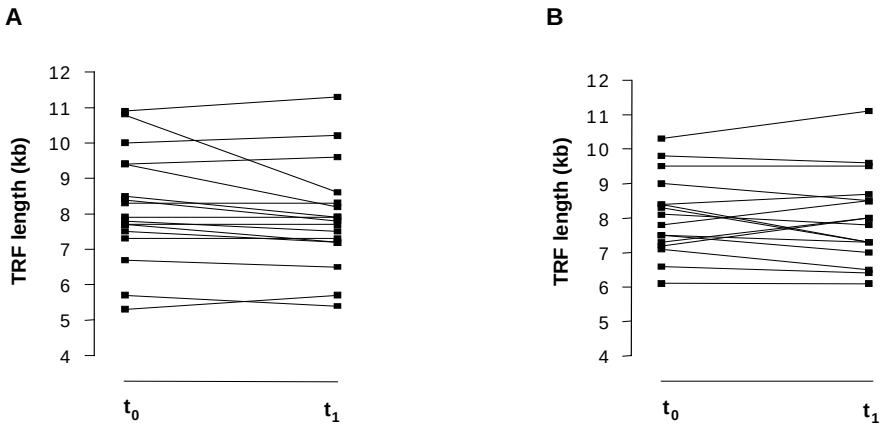


Figure 1:

Haematological recovery

The open bar indicates the standard-dose group, the black bar the high-dose group. A (*) indicates a significant difference in paired samples compared to t_0; a (+) indicates a significant difference between groups at that time point. On the X-axis blood sampling times t_0 (prior to chemotherapy), t_1 (5 months after chemotherapy) and t_2 (9 months after chemotherapy) are indicated. On the Y-axis, the following values are indicated with mean + SD: **A**: leukocytes (10^9 l^{-1}); **B**: platelets (10^9 l^{-1}; **C**: haemoglobin (mmol l^{-1}) and **D**: MCV (fL).

Figure 2:



Paired TRF samples

A: X-axis: standard-dose group samples, at t_0 and t_1; Y-axis: TRF length (kilobase, kb). **B**: X-axis: high-dose group samples, at t_0 and t_1; Y-axis: TRF length (kb).

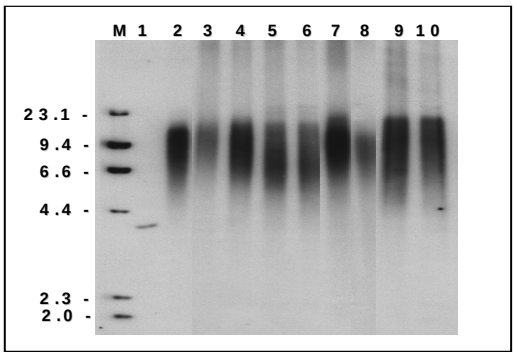


Figure 3:

Representative example of blot to measure TRF length

M: marker; lane 1: plasmid control; lane 2: leukocyte control healthy volunteer; lanes 3-10: paired patient samples; lanes 3 and 4: t_0 and t_1 sample, standard-dose treatment (Δ TRF length -0.7 kb); lanes 5 and 6: t_0 and t_1 sample, standard-dose treatment (Δ TRF length 0 kb); lanes 7 and 8: t_0 and t_1 sample, high-dose treatment (Δ TRF length -1.1 kb); lanes 9 and 10: t_0 and t_1 sample, high-dose treatment (Δ TRF length + 0.3 kb).

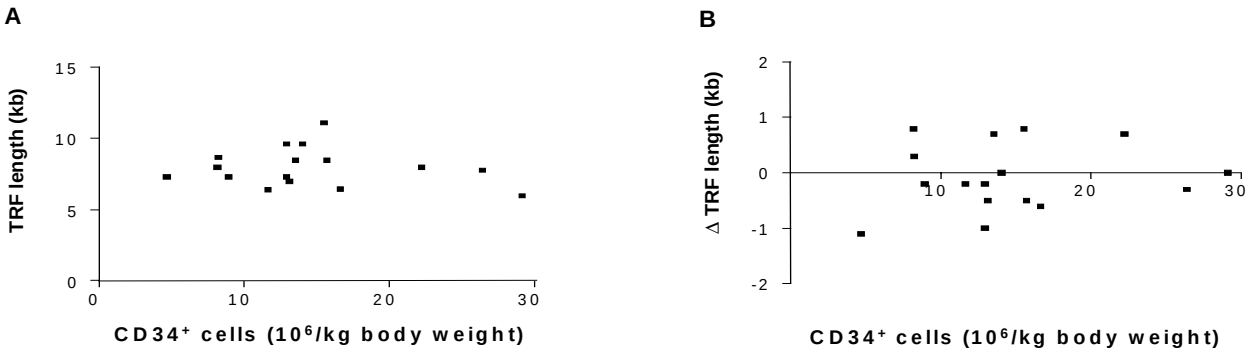


Figure 4:

TRF length and CD34+ cell numbers

On the X-axis, the number of reinfused CD34+ cells (10^6per kg body weight) is indicated; on the Y-axis the following values are indicated: **A**: TRF length (kb): measured value at t_1 after high-dose treatment, **B**: Δ TRF length (kb): calculated difference between TRF values at t_0 and t_1 of high-dose treatment.

Death receptors in primary breast cancer

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Submitted

ABSTRACT

Introduction: Death receptors Fas (receptor for Fas Ligand, FasL), and DR4 and DR5 (receptors for TNF-Related Apoptosis Inducing Ligand, TRAIL) in primary breast tumors, are likely related to apoptosis induction. They may be of interest for breast cancer treatment. Therefore, the presence of death receptors (Fas, DR4 and DR5), and Fas Ligand (FasL) was evaluated using immunostaining. Proliferation (Ki-67), apoptotic index, and apoptosis inhibition (Bcl-2) were evaluated using immunostaining. In addition, since death receptors may be up-regulated by estrogen deprivation, these parameters were evaluated in a series of tumors after pre-operative anti-estrogen therapy. Patients and methods: Primary breast tumors from 35 pre-menopausal, progesterone receptor (PR) positive, breast cancer patients were obtained. Nineteen patients had not received pre-operative treatment; 16 patients had received pre-operative tamoxifen (40 mg p.o., daily for 7-10 days), and LH-RH agonist gosereline (3.6 mg s.c. injection, once). Tumors were stained for Fas, FasL, DR4, DR5, Ki-67 and Bcl-2, immunohistochemically. Apoptotic cell counts were studied microscopically and expressed as apoptotic index (% of apoptotic cells). In five normal breast samples, Fas, FasL, DR4 and DR5 staining was assessed. Results: Tumors were positive for Fas (38%), DR4 (71%), DR5 (91%), or for combinations; all tumors were positive for at least one of these receptors. DR4 or DR5 staining was present in 97% of tumors. In all tumors, DR4 staining was positively correlated with DR5 staining ($p=0.006$), and Bcl-2 staining ($p=0.018$); FasL and apoptotic index were inversely correlated ($p=0.008$). No differences in the evaluated parameters were observed in tumors with or without anti-estrogen treatment. Normal breast samples were positive for Fas (100%), negative for DR4 (100%) and mostly negative for FasL and DR5 (both 75%). Conclusion: Death receptors, DR4 and DR5 in particular, are abundantly present immunohistochemically in primary breast tumors of PR+ pre-menopausal patients, while they are mostly absent in normal breast tissue. Short-

term anti-estrogen treatment does not further increase this. These results indicate that TRAIL could possibly be a tumor-specific future treatment for PR+ breast cancer.

INTRODUCTION

The role of the tumor necrosis factor (TNF) family in inducing programmed cell death or apoptosis in breast cancer cells has gained increasing interest in recent studies. Both Fas Ligand (FasL) and TNF-Related Apoptosis Inducing Ligand (TRAIL) were shown to have increased expressions in malignant breast tissue compared to benign counterparts (1-7). The expression of Fas (the receptor of FasL) was found to be decreased in many malignant (compared to benign) breast tumors (8), which was associated with a worse clinical outcome (2). A high FasL:Fas mRNA ratio was related to reduced disease-free survival and increased mortality in breast cancer patients (9). Upregulation of FasL or TRAIL may prevent tumor cell death by evading the immune system (3, 4, 10). Down-regulation of the Fas receptor, which upon binding with FasL induces apoptosis, may have a similar effect. Whether TRAIL receptors DR4 and DR5 are also affected in the course of malignant progression, has not been described yet in primary breast tumors. It might be suggested that the expression of death receptors and their ligands play an important role in breast cancer cells. The presence of death receptors in primary breast tumors is highly interesting, in view of the potential treatment possibilities for breast cancer. Treatment with TRAIL is of particular interest, as TRAIL induced apoptosis presumably takes place in a fairly tumor specific fashion (11-13), with potentially less systemic toxic effect in-vivo compared to FasL (14). The sensitivity for apoptosis induced by the anti-Fas antibody or TRAIL can be further enhanced by treatment with chemotherapeutic agents (15-17) in-vitro, as well as with radiation (14) in an animal model with a tumor from the human breast cancer cell line MCF-7. Also in response to deprivation of estrogen (the primary stimulant for breast cell proliferation (18, 19) up-regulation of death receptors Fas (20-22), and possibly DR5 (23, 24) was described in an animal model (20) or in-vitro (21-24). In pre-menopausal women with breast cancer, a combination of the (partial) estrogen

antagonist tamoxifen and the luteinizing hormone-releasing hormone (LH-RH) agonist gosereline induces estrogen deprivation comparable to castrate levels (25). This estrogen blockade has recently gained increasing interest (26), as the optimal result of adjuvant treatment in pre-menopausal hormone receptor positive breast cancer patients can be obtained by a combination of chemotherapy and estrogen deprivation (27).

In this light, the current study was performed to evaluate the presence of death receptors Fas, DR4 and DR5, as well as FasL in primary progesterone receptor positive breast tumors, using immunohistochemical techniques. Results were related to expression of the anti-apoptotic protein Bcl-2, and apoptotic- and proliferative index (Ki-67 staining with the antibody MIB-1). In addition, we examined whether the above mentioned parameters were affected by estrogen deprivation in a separate group of primary breast tumors, after pre-operative estrogen blockade (effected by partial estrogen antagonist tamoxifen and luteinizing hormone releasing hormone (LH-RH) agonist gosereline). To our knowledge, this is the first report including immunohistochemical assessment of death receptors DR4 and DR5 in primary breast cancer.

PATIENTS, MATERIALS AND METHODS

Patients

Archival primary breast cancer tissue was retrieved from pre-menopausal, progesterone-receptor (PR) positive, breast cancer patients. Staging of patients was performed according to the TNM system (Union Internationale Contre le Cancer, 1997). Samples from patients without pre-operative treatment were obtained from the Isala Clinics, Zwolle (the Netherlands), and the University Hospital Groningen (The Netherlands). Surgical treatment of these patients was performed in 1994 (Isala Clinics), or between April 1997 and August 1999 (University Hospital Groningen). From these time intervals, samples from all eligible patients (e.g.: pre-menopausal, PR+) were included. This group will be referred to as group I.

In addition, archival primary breast cancer tissue was retrieved from pre-menopausal, PR+ breast cancer patients who had received pre-operative treatment with 40 mg oral tamoxifen daily for 7-10 days (the interval between diagnosis and surgery), and a subcutaneous gosereline 3.6 mg injection, once. From all eligible patients (e.g.: pre-menopausal, PR+) who had received this treatment between February 1990 and May 1996 (Isala Clinics, Zwolle), samples were included. This group will be referred to as group II.

Control archival normal breast samples (n=5) were obtained from pre-menopausal women, operated upon between 1996 and 1999 (University Hospital Groningen). Four samples were derived from patients after prophylactic ablation of the breast, and one sample was obtained after breast reduction; this sample was not found to contain epithelial hypertrophy.

Immunohistochemistry

All primary tumor samples were fixed in 4% formalin and paraffin embedded; 4 µm sections were prepared. The presence of carcinoma in the sections was checked using standard hematoxylin-eosin (H&E) staining on parallel sections.

Prior to staining, slides were deparaffinized and dehydrated. Air-drying was performed for at least 15 min prior to antigen-retrieval, except prior to DR4 staining. Antigen retrieval was performed by means of autoclave treatment with heating 3 times for 5 min at 115 ºC in blocking reagent (2% block + 0.2% SDS in maleic acid, pH=6.0, Roche, Mannheim, Germany) for Fas, FasL, Bcl-2 and Ki-67 (MIB-1 antibody) staining; or by means of adding slides to 10mM citric acid monohydrate (Merck, Darmstadt, Germany) solution in demineralized water, pH=6.0, and subsequent microwave treatment at 100°C for 8 min at 700W for DR5 staining.

Endogenous peroxidase activity was blocked by treatment of slides with 1% peroxide in phosphate buffered saline solution (PBS, 0.14 M NaCl, 2.7 mM KCl, 6.4 mM $Na_2HPO_4.2H_2O$, 1.5 mM KH_2PO_4, pH 7.8) for 30 min. Slides were then washed twice with PBS. Slides were pre-incubated with 1% AB serum, 1% bovine serum albumin (BSA, Life Technologies, Breda, The Netherlands) in PBS, for 30 min. For DR5 staining, slides were additionally pre-incubated with avidin and biotin blocking reagent (Vector laboratories, Burlingame, CA).

All antibodies were supplied by DAKO (Glostrup, Denmark) except if indicated differently, and they were diluted in PBS, 1% BSA. Slides were then stained for 60 min with anti-FasL antibody (Transduction Laboratories, Lexington KY), diluted 1:160; anti-Fas antibody (CH11, Upstate Biothechnology, Waltham, MA), diluted 1: 100; anti-DR4 antibody (Santa Cruz, Santa Cruz, CA), diluted 1: 50; anti-DR5 antibody (Oncogene, Cambridge, MA), diluted 1:100; anti-Bcl-2 antibody, diluted 1:50, or anti-Ki67 antibody (MIB-1, Immonotech, Marseille, France) diluted 1:400. For the negative control, no antibody was added to the PBS. Positive controls were (tumor) tissue

samples, found on previous occasions to stain positive: liver tissue for Fas, FasL and DR5; colon cancer tissue for DR4 and Ki-67; cervical cancer tissue for Bcl-2.

Slides were washed with PBS three times, and then incubated for 30 min with the secondary antibody Rabbit Anti Mouse-bio, diluted 1:300 (for Fas, FasL and DR4), Rabbit Anti Mouse-peroxidase diluted 1:300 (for Ki-67 and Bcl-2) or Swine Anti Rabbit-bio diluted 1:300 (for DR5), followed by washing in PBS 3 times. Slides were then incubated for 15 min with streptavidin, diluted 1:300 (for Fas, FasL, DR4 and DR5), or Goat Anti Rabbit-peroxidase streptavidin, diluted 1:300 (for Ki-67 and Bcl-2), followed by washing in PBS 3 times. For the peroxidase reaction, di-aminobenzidine (DAB: Sigma Cemical CO., St. Louis, MO), 25 mg was dissolved in 50 mL imidazol solution (1 mg/mL PBS), to which 50 µL 30% peroxide was added prior to use. After this staining, slides were washed with demineralized water. Counter-staining was performed with hematoxylin for 2 min, after which slides were washed with regular water for 5 min. Slides were subsequently dehydrated, air-dried, and embedded in mounting medium.

Staining was scored by two individual examiners: in line with Mottolese et al. (2) for Fas and FasL: <10% of tumor cells staining positive was regarded negative; 10-50% of cells staining positive was regarded heterogeneous and > 50% of cells staining positive was regarded positive. The DR4 staining was scored in a similar way as Fas and FasL. Ki-67 staining with MIB-1 was scored in percentage of stained nuclei (in categories of 10%); Bcl-2 and DR5 staining was scored as no staining (0), weak staining (1), moderate staining (2) and strong staining (3). Apoptotic index was determined in H&E slides as the percentage of cells with nuclei showing clumping of chromatin. Of each tumor, at least 3 times 200 cells were scored.

Staining for PR and estrogen receptor (ER) was performed (for confirmation of progesterone and estrogen receptor status) in all samples with a standardized method. After deparaffinization, antigen retrieval was performed as described above for Fas and

FasL staining. An automated immunostainer was used (Ventana Medical Systems Inc., Tucson, AZ), with the anti-PR or anti-ER antibody (Ventana Medical Systems Inc., Tucson, AZ). Slides were subsequently dehydrated, air-dried, and embedded in mounting medium. Samples were scored according to a standard protocol, accounting for the proportion of stained cells as well as the intensity of staining, and samples were considered positive when the overall score was ≥ 3 (28). The positive or negative distinction was used for further analyses.

Statistics

Statistics were performed with SPSS software. The Mann-Whitney U-test was used for comparing immunohistochemical staining of Fas, FasL, DR4, DR5, Ki-67, Bcl-2 and apoptotic index, between group I and group II. Age of patients was compared between group I and group II by means of the Student's t-test. Correlations between Fas, FasL, DR4, DR5, Ki-67, Bcl-2 staining and apoptotic index, and tumor stage, were determined with the Pearson correlation test. P-values ≤ 0.05 were considered statistically significant.

RESULTS

Patients

Patient characteristics are shown in table 1.

Immunohistochemistry

Immunohistochemical staining and apoptotic index are shown in table 2. All stainings were found to be cytoplasmatic, except with the Ki-67 staining with the MIB-1 antibody (nuclear). An example of DR4 and DR5 staining is shown in figure 1. For Fas, FasL and DR4, intensity of staining within the areas comprising malignant tumor, was heterogeneous. Therefore, for scoring these stainings, the percentage of malignant tumor that stained positively was used. The intracellular FasL staining was found to be vesicle-like. The DR5 and Bcl-2 staining was usually homogeneous, allowing scoring for intensity. Ki-67 staining with MIB-1 was nuclear and vesicle-like. Both DR4 and DR5 staining were usually also detected in the normal tissue, surrounding the tumor. This staining was restricted to the epithelial cells, and was less intense than in the neighboring tumor tissue. A similar staining pattern was found with FasL, but with an outspoken distinction between normal surrounding tissue (negative) and malignant parts (positive). In contrast, the Fas staining was generally present in normal epithelial cells, while staining intensity was decreased in the malignant parts. One tumor sample, containing a small part of ductal carcinoma in situ (DCIS) next to an invasive tumor, was found to be positively stained in the normal part and the DCIS part, but was negative in the invasive tissue. Control normal breast samples were generally found Fas positive (5 of 5 samples), FasL negative (3 of 4 samples negative, one sample not assessable), DR4 negative (5 of 5 samples) and DR5 negative (3 of 4 samples negative, 1 sample weakly positive, one sample not assessable).

Between group I and II (with or without anti-estrogen treatment), no statistical differences were found for any of the parameters. This indicates that the investigated parameters were unchanged by the short term administration of anti-estrogen treatment. When both groups were combined, 38% of tumors were Fas-positive and 80% was FasL-positive. Positivity for DR4 (71% of tumors) and DR5 (91% of tumors) was considerable, as was the presence of combinations of death receptors (DR4 and DR5: 68% of tumors; DR4 and DR5 and Fas: 24% of tumors). Nearly all (97%) tumors were positive for either DR4 or DR5. All tumors were positive for at least one death receptor. The occurrence of combinations of these death receptors is shown in table 3. As no difference regarding the stainings was found between the two groups, both groups were shown combined.

Concomitant DR4- and DR5-staining in the combined groups, was found to be positively correlated to apoptotic index ($r=0.344$, $p=0.046$), but the combination of DR4 and DR5 and Fas, or Fas and FasL was not correlated to apoptotic index. A positive correlation was observed between DR4- and DR5-staining ($r=0.465$, $p=0.006$, reflected in figure 2), and between DR4- and Bcl-2 immunoreactivity ($r=0.402$, $p=0.018$, reflected in figure 3). A negative correlation was observed between FasL staining and apoptotic index ($r=-0.449$, $p=0.008$, figure 4).

DISCUSSION

An increasing number of studies indicate a role for death receptors and their ligands in breast cancer. For Fas and FasL in particular, changes in expression have been described, in the course of malignant progression. Fas was found to be decreased and FasL increased, compared to benign counterparts (1-3, 5-9). In most studies (1-4, 6-9), but not all (5), these changes have been attributed to the advantages they create for tumor cells to evade immune responses. TRAIL expression in tumor cells may also present an immunologic advantage (10). While one study reported the presence of the ligand TRAIL in primary breast tumors (7), the status of the death receptors DR4 and DR5 in primary breast tumors was unknown so far. Assessment of these receptors in tumors is particularly interesting in view of the potential use of TRAIL in the treatment of breast cancer. TRAIL induced apoptosis presumably takes place in a fairly tumor specific fashion, through a family of agonistic (DR4 and DR5) and antagonistic receptors (DcR1 and DcR2) (11-13). This aspect renders the clinical use of recombinant human rhTRAIL of potential interest. Furthermore, rhTRAIL does not appear to have the systemic toxic effect in-vivo as treatment with FasL (14), although in-vitro work suggests that hepatoxicity by a polyhistidine-tagged recombinant TRAIL in humans may occur (29). This aspect needs further attention (30). The recent finding that native-sequence (non-tagged), clinical grade rhTRAIL had minimal toxicity towards human hepatocytes and absence of hepatotoxicity in cynomolgus monkeys following repeated administration of intravenous properly folded rhTRIAL is reassuring (31). It may well be that clinical-grade rhTRAIL is suitable for investigation in cancer patients (31). In view of the potential use of rhTRAIL to induce apoptosis in breast cancer, means to upregulate the TRAIL receptors DR4 and DR5 are of interest. Possibly, estrogen deprivation may be one of those means (23, 24). Other TNF receptor family members Fas (20-22) and TNFR1 (32) were also shown to be up-regulated in

breast cancer cells by treatment with some anti-estrogens (20-22, 32), but not with others (32, 33). The current immunohistochemical study was performed to gain more information on the presence of death receptors Fas, DR4 and DR5 and Fas ligand in primary breast tumor samples. Also, proliferation and apoptosis were examined. In addition, the effects of estrogen blockade by means of tamoxifen and gosereline on these parameters was examined.

In line with other studies (1-7), we found a high percentage of FasL positive tumors (80%), and a lower percentage (38%) of Fas positive tumors. Most Fas positive tumors were also positive for FasL (12/13). DR4 and DR5 staining was found present in the vast majority of tumors. Most tumors were DR4 positive (71%), while other tumors showed a more heterogenous staining pattern, or were negative. A large majority of tumors was DR5 positive (91%), and strong staining was found in a number of these samples. In addition, the majority of tumors were actually positive for both DR4 and DR5 (68%), and these stainings were correlated. The simultaneous presence of DR4 and DR5 was also found to be correlated to apoptotic index. Nearly all tumors (97%) were positive for either DR4 or DR5. These results indicate that DR4 and DR5 are abundantly present in primary breast tumors and could serve as a possible target for TRAIL induced apoptosis. In-vitro data have indicated the presence of these receptors in breast cancer cell lines (16), but sofar this has not been confirmed in primary breast cancer. In normal pre-menopausal breast samples, we found no samples positive for DR4, while one sample was weakly positive for DR5. Although the number of samples was small, this could possibly be an indication of the tumor specificity of these receptors, which may increase the clinical feasibility of rhTRAIL based therapy in breast cancer. However, the role of their antagonistic receptors (DcR1 and DcR2) in primary breast tumors in this respect, remains to be clarified. We also found correlations between FasL and apoptosis, and DR4 and Bcl-2 staining. The correlation between FasL staining and apoptotic index was negative, and

may therefore support the notion that increased FasL expression can possibly protect breast cancer tissue from apoptosis induced by Fas bearing immune cells (1, 3, 4, 6, 7). Since TRAIL induced apoptosis can be inhibited by increasing Bcl-2 levels (34), a positive correlation between DR4 and Bcl-2 staining may suggest a protective role for Bcl-2 in breast cancer. The need for such protection may follow from the fact that the majority of breast cancers are DR4 positive (this study), but may also be TRAIL positive (7): this could possibly imply the functional, or membrane related, DR4 receptor in our samples.

In this study, short-term anti-estrogen treatment with gosereline and tamoxifen was not shown to induce clear effects on the examined parameters: no indications were found for an acute increase of apoptosis due to this treatment. The fact that not all tumors in the anti-estrogen treatment group were ER+ (in spite of being all PR+) was presumably induced by the prior treatment itself (35). The Bcl-2 staining intensity tended towards a slight decrease after anti-estrogen treatment in our samples, but this was not significant. Anti-estrogen treatment induced a swift Bcl-2 decrease in-vitro (36-38), and maximal apoptosis after 48 h in an animal model (39). However, in the human in-vivo setting, effects of neo-adjuvant anti-estrogen treatment on Bcl-2 and apoptosis were shown after 14 days to 3 months (35, 40-42). It may be suggested that the duration of tamoxifen administration in our study (7 to 10 days) in combination with gosereline, was too short for detecting effects on proliferation and apoptosis. In addition, it has been described that the combination of tamoxifen and gosereline may actually induce an increase in serum estradiol levels, prior to a reduction of hormones to castrate levels after 3 weeks (25). This hormonal fluctuation could have affected parameters such as Bcl-2 staining, in the time-interval of the present study. Furthermore, this might also be related to the fact that no inhibition of FasL by tamoxifen was found in our in-vivo setting, in contrast to recent in-vitro data (43). In light of this, the in-vivo effects of anti-estrogen treatment on apoptosis

markers and death receptors, and its time-dependency in particular, remain to be clarified. To this end, preferably pre-and post-treatment samples will have to be used in larger studies. Particularly in view of the increasing interest for anti-estrogen treatment in pre-menopausal breast cancer patients (26, 27), evaluation of in-vivo effects on apoptosis and death receptors may help improve breast cancer treatment.

In conclusion, death receptors, and DR4 and DR5 in particular, are abundantly present in the majority of primary breast tumors, while they are mostly absent from normal breast tissue. Short-term anti-estrogen treatment did not increase this further. These results indicate that rhTRAIL treatment could possibly be a tumor-specific treatment in hormone receptor positive breast tumors.

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TABLE 1: **Patient characteristics**

		Group I (n=19) no treatment	**Group II** (n=16) anti-estrogen treatment
Mean age in years (range)		44 (34-52)	46 (32-52)
Disease stage	I	7	0
	II	11	13
	III	0	2
	IV	1	1
PR (n)	positive	19	16
ER (n)	positive	19	13
Tumor type	ductal	18	13
	lobular	1	2
	DCIS	-	1

TABLE 2: **Immunohistochemical staining and apoptosis**

		Group I (n=19) No treatment (tumor no./total no.; percentage)		**Group II** (n=16) Anti-estrogen treatment (tumor no. /total no.; percentag		
Fas	positive	7/18	(39 %)	6/16	(38 %)	
	negative/ heterogeneous	11/18	(61 %)	10/16	(62 %)	
	n.a.*	1/19				
Fas L	positive	16/19	(84 %)	12/16	(75 %)	
	negative/ heterogeneous	3/19	(16 %)	4/16	(25 %)	
DR4	positive	14/19	(74 %)	11/16	(69 %)	
	negative/ heterogeneous	5/19	(26 %)	5/16	(31 %)	
DR5	strong	3/18	(17%)	1/16	(6%)	
	moderate	8/18	(44%)	4/16	(25%)	
	weak	7/18	(39%)	8/16	(50%)	
	negative			3/16	(19%)	
	n.a.	1/19				
Bcl-2	strong	5/18	(28%)	2/16	(13%)	
	moderate	8/18	(44%)	5/16	(31%)	
	weak	5/18	(28%)	5/16	(31%)	
	negative			4/16	(25%)	p=0.09
	n.a.	1/19				
Ki-67	% (range)	mean 23 (0-60)		mean 16 (0-50)		
Apoptosis	% (range)	mean 1.3 (0-6)		mean 1.7 (0-5)		

* n.a.: not assessable

TABLE 3: **Combinations of death receptors (group I and II)**

(in 34 assessable tumors*)

		No. of tumors (percentage)	
Fas and FasL	Positive	12	(35%)
DR4 and DR5	Positive	23	(68%)
DR4 or DR5	Positive	33	(97%)
DR4, DR5 and Fas	Positive	8	(24%)
All death rec.	Negative	0	

***** combinations of death receptors could be determined in 34 tumors, as Fas and DR5 staining was not assessable in one tumor (see: table 2).

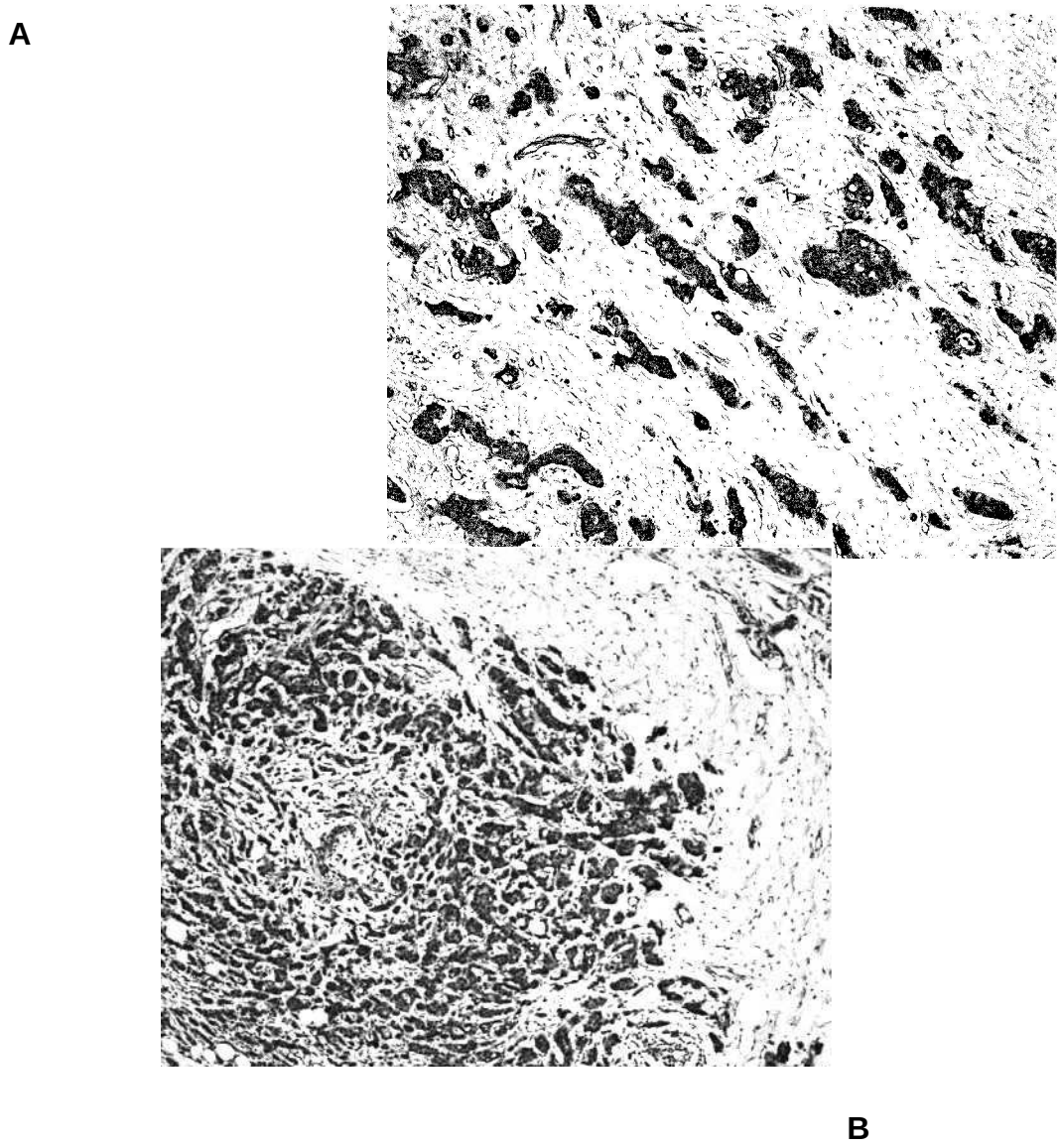

Figure 1: DR4 and DR5 staining
A: example of DR4 staining, magnification 10x10;
B: example of DR5 staining, magnification 10x10.

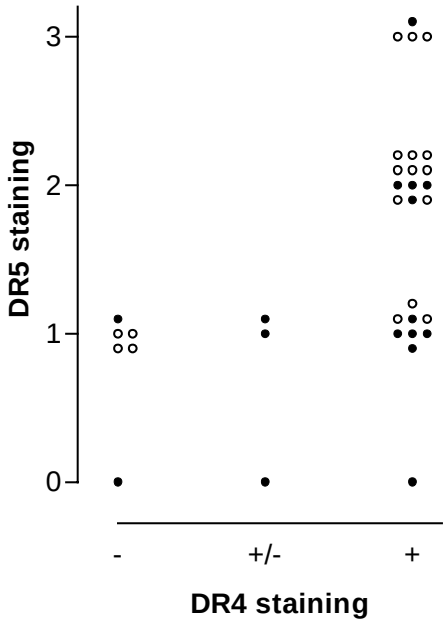

Figure 2: **relation of DR4 and DR5 staining**
X-axis: DR4 staining (negative, heterogeneous and positive); Y-axis: DR5 staining (0: negative, 1: weak, 2: moderate, and 3: strong). Open dot: no anti-estrogen treatment; solid dot: anti-estrogen treatment. DR4 and DR5 are positively correlated for samples of both groups: r=0.465, p=0.006. No difference in either DR4 or DR5 staining is observed between both groups.

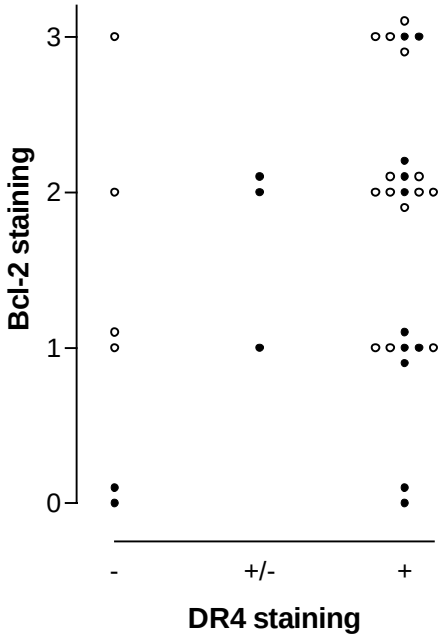

Figure 3: relation of DR4 and Bcl-2 staining
X-axis: DR4 staining (negative, heterogeneous and positive); Y-axis: Bcl-2 staining (0: negative, 1: weak, 2: moderate, and 3: strong). Open dot: no anti-estrogen treatment; solid dot: anti-estrogen treatment. DR4 and Bcl-2 are positively correlated for samples of both groups: r=0.402, p=0.018. No difference in either DR4 or Bcl-2 staining is observed between both groups.

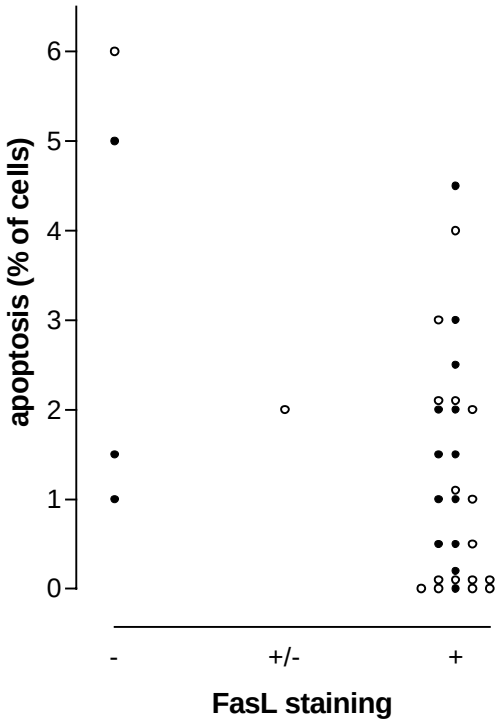

Figure 4: relation of FasL staining and apoptotic index
X-axis: FasL staining (negative, heterogeneous and positive); Y-axis: percentage of apoptotic cells (apoptotic index). Open dot: no anti-estrogen treatment; solid dot: anti-estrogen treatment. FasL and apoptosis are negatively correlated for samples of both groups: r=-0.449, p=0.008. No difference in either FasL or apoptotic index is observed between both groups.

Chapter 11

Summary and future perspectives

Optimizing breast cancer treatment: detection of micrometastatic disease

Breast cancer patients without apparent distant metastases at the time of primary tumor removal, may later suffer from a distant relapse, indicating the presence of occult micrometastases at the time of diagnosis. Sensitive methods to detect micrometastatic breast cancer may be helpful in optimizing treatment for breast cancer patients. It may facilitate the selection of patients for early systemic 'adjuvant' therapy and the evaluation of response to adjuvant therapy. In early stage breast cancer, the options to give increasingly aggressive treatment approaches (such as high-dose chemotherapy followed by a transplantation of peripheral blood stem cells (PBSC)), are expanding. In this regard, an optimal selection of those patients who are likely to benefit, and those who are not but will suffer from the harsh side-effects, is becoming more and more important. Detection of tumor cells in PBSC would retrospectively allow evaluation of the impact of transplanting a minimal amount of these cells into the patient, as a contaminant of the PBSC material. Furthermore, if minimal amounts of tumor cells would prove detectable, (in-vitro) methods for removing these tumor cells could be evaluated.

In chapter 2, an overview is given regarding micrometastatic breast cancer, with special attention to potential tumor cell contamination in stem cell harvests, and the impact of hematopoietic growth factors. In chapter 3, the use of epithelial glycoprotein-2 (EGP-2) as a membrane-marker for detection of breast cancer cells, by means of a quantitative reverse-transcriptase polymerase chain reaction method (qRT-PCR) as well as immunostaining was studied. EGP-2 is a pan-carcinoma tumor-associated, epithelial-tissue specific marker, and is universally expressed in breast cancer. The results were compared with the use of the commonly employed cytokeratin 19 (CK19) marker. The qRT-PCR was performed on breast tumors to determine a 'cut-off point' for EGP-2 expression in blood samples. The expression of EGP-2 in breast tumors was found to vary 100-fold. It was concluded that PCR

based methods for detecting breast cancer cells in blood may be hampered by this variable expression of tumor-associated tissue-specific markers in breast cancer tumors.

In chapter 4, the detection of micrometastases was evaluated in a series of corresponding primary breast tumor tissue, sentinel lymph nodes and peripheral blood samples. Immunostaining and real time qRT-PCR was performed to detect low amounts of EGP-2 and CK19 positive cells. The detection limit was proven to be as low as one cell, using the breast cancer cell line MCF-7 as a standard, in one to two million leukocytes with these methods. Control nodes from patients without cancer showed aspecific CK19 staining of dendritic reticulum cells, but were qRT-PCR negative. Control blood samples from healthy volunteers were all negative. Primary tumor samples from 58 patients were all positive with immunostaining, but showed a wide variation in EGP-2 ($>10^4$ fold) and CK19 mRNA expression ($>10^3$ fold). Sentinel nodes from 16 patients were found to be tumor positive after routine hematoxylin-eosin (H&E) staining or EGP-2 and CK19 directed immunostaining. A correlation was found between qRT-PCR results and the presence of tumor in sentinel nodes, but also false positive and false negative results were observed. Peripheral blood samples (n=149), collected perioperatively, were all negative with immunostaining, whereas 19 patients (one third of patients) had one or more qRT-PCR positive blood samples. It was concluded that again, primary tumor cells show a wide variation of EGP-2 or CK19 mRNA expression. Since not all tumor cells express these markers simultaneously to a high extent, both markers may be used separately in qRT-PCR for adequate detection sensitivity. In sentinel nodes, detection of tumor presence using immunostaining appears more sensitive and specific than using routine H&E staining or qRT-PCR. In peripheral blood, no samples were found to contain tumor cells using immunostaining, while one third of patients had qRT-PCR positive samples; this could possibly indicate a higher

sensitivity of the latter procedure. The clinical value of these findings will have to be evaluated by long-term follow-up, in a large series of patients.

An important finding was that the negative controls remained negative with this real time qRT-PCR method. Therefore, the need to combine peripheral blood and primary tumors, for establishing a 'cut-off' point for expression in peripheral blood for each individual patient, was not so urgent with this qRT-PCR method, in contrast to the older qRT-PCR method (described in chapter 3). Therefore, in chapter 5, the real-time qRT-PCR methodology was applied to sequential blood- and PBSC samples (a total of 174 samples) of 59 breast cancer patients, for detection of tumor cells. Samples were obtained prior to-, during, and after treatment, from patients randomized to receive standard-dose chemotherapy, or high-dose chemotherapy and PBSC transplantation. With immunostaining for EGP-2, two samples (during treatment: one blood and one PBSC sample) from two patients were found tumor positive. With qRT-PCR, one blood sample was found positive for CK19 mRNA expression, and 12 samples (5 PBSC samples, and 7 blood samples) from 12 patients were positive for EGP-2 mRNA expression. One patient had one immunostaining and one qRT-PCR positive sample, but at different time-points. The clinical implications of these findings will have to be clarified with further follow-up data in a large series of patients.

Optimizing breast cancer treatment: purging of micrometastatic disease

In chapter 6, an in-vitro method was described to purge (i.e. to remove) minimal quantities of tumor cells from PBSC, as tumor cell contamination of the stem cells is a potential source of renewed tumor development. Specific carcinoma cell kill can be obtained by retargeting activated (cytotoxic) T cells with bispecific antibody BIS-1, directed against EGP-2 and T cell receptor CD3. Activation of T cells, also present in PBSC material, and retargeting of these T cells to tumor cells by BIS-1, was used

to initiate a purging process in the PBSC material. Activation of T cells was performed by culturing PBSC in phosphate buffered saline (as control), interleukin-2, anti-CD3 receptor antibody or a combination of interleukin-2 and anti-CD3 receptor antibody. It was shown that prior to activation, breast cancer patients PBSC material contained higher levels of CD8+ T cells (cytotoxic T cells), compared to peripheral blood from healthy volunteers ($p<0.05$). The potential of PBSC material to sustain tumor cell lysis was increased after all prior activations, and was further enhanced by BIS-1. Maximal BIS-1 effect was observed after 72 hours of anti CD3 antibody activation of peripheral blood stem cells, inducing a >3 log depletion of tumor cells. This means that more than 99.9% of tumor cells was killed. Hematopoietic colony formation was not affected by prior anti-CD3 receptor antibody activation, and/or BIS-1. Therefore, it was concluded that specific tumor cell lysis by peripheral blood stem cells can be obtained in-vitro by anti-CD3 receptor antibody activation and BIS-1 retargeting of T cells of the PBSC itself, without affecting hematopoietic colony formation of stem cells.

BIS-1 mediated purging was also applied in a different setting, in an in-vitro model to purge carcinoma cells from cryopreserved ovarian tissue. The background of this study, described in chapter 7, was that aggressive chemotherapy and/or radiotherapy for the treatment of cancer can lead to impaired fertility in female patients. Cryopreservation and autografting of ovarian tissue is a promising new method for conserving their fertility, but tumor cell contamination of the autograft may form a problem. Therefore, we evaluated the survival of MCF-7 tumor cells, after co-incubation with activated lymphocytes and BIS-1, in the presence or absence of a suspension of thawed human ovarian tissue. It was shown that MCF-7 cells were increasingly more killed with increasing lymphocyte to MCF-7 cell ratio's in the presence of BIS-1. Adding ovarian tissue did not negatively affect tumor cell kill. Importantly, ovarian tissue included morphologically intact follicles that proved

to be preserved after this purging procedure. It may be suggested that this method may contribute in the future to the safe replacement of ovarian tissue in female cancer survivors.

More ways to optimize breast cancer treatment

In chapter 8, the impact of recombinant human granulocyte-colony stimulating factor (rhG-CSF) was studied for prevention of febrile leucopenia (bone marrow depression combined with fever), induced by chemotherapy. RhG-CSF is known to increase granulocyte counts, thus ameliorating the patients response to possible infectious pathogens. A prospective randomized trial was performed, in which 40 stage IV breast cancer patients undergoing intermediate high-dose chemotherapy (cyclophosphamide, 5-fluorouracil plus epirubicin or methotrexate), received either rhG-CSF or a combination of ciprofloxacin and amphotericin B. In the group receiving prophylactic rhG-CSF, seven of 18 patients (after 10/108 courses) showed febrile leucopenia; in the group receiving ciprofloxacin and amphotericin B, seven of 22 patients (7/98 courses) (p=N.S). Also median hospitalization duration and associated costs were not different. However, rhG-CSF itself was 6.6 times more expensive per course than ciprofloxacin and amphotericin B. It is concluded, that in the present study a combination of ciprofloxacin and amphotericin B has similar efficacy as rhG-CSF in preventing febrile leucopenia, and is more cost-effective.

In chapter 9, the possible induction of an accelerated 'aging process' in the hematopoietic stem cell compartment by adjuvant high-dose chemotherapy and PBSC transplantation was evaluated. Accelerated aging of hematopoietic stem cells may have important undesirable long-term effects, that could be clinically relevant in patients with a relatively good prognosis. Telomere length is a marker for cell lineage age, as it decreases with every cell division. Therefore, leukocyte telomere

length and telomerase activity were studied before and after treatment in breast cancer patients randomized to receive either standard-dose chemotherapy (17 patients), or high-dose chemotherapy and PBSC transplantation (16 patients). Haemoglobin, MCV, leukocyte- and platelet numbers were assessed prior to (t_0), 5 months after (t_1) and 9 months after chemotherapy (t_2). These parameters of haematological reconstitution were decreased at t_1/t_2 compared to t_0 (high-dose: all parameters; standard-dose: leukocytes/platelets), and all parameters were lower after high-dose than standard-dose treatment at t_1. Paired individual leukocyte samples of t_0 and t_1 showed telomere length change ranging from +0.8 to –2.2 kb, with a decreased telomere length in 9 patients in both groups (N.S.). Telomerase activity was below detection limit in leukocyte samples of t_0 and t_1. It was concluded, that standard- and high-dose chemotherapy negatively affect haematologic reconstitution. Although telomere length was changed in individual patients, the overall conclusion is that no support for accelerated telomere loss in stem cells due to haematologic proliferative stress is found in this setting.

In chapter 10, another aspect of breast cancer was studied. Death receptors Fas (receptor for Fas Ligand, FasL), and DR4 and DR5 (receptors for TNF-Related Apoptosis Inducing Ligand, TRAIL) in primary breast tumors, are likely related to the induction of apoptosis, i.e. regulated cell death. They may be of interest for breast cancer treatment. Therefore, the presence of death receptors (Fas, DR4 and DR5), and Fas Ligand (FasL) was evaluated using immunostaining in breast tumors. Anti-apoptotic protein Bcl-2 immunostaining, apoptotic- and proliferative index (Ki-67 immunostaining with its antibody MIB-1) were also evaluated. In addition, since in-vitro reports have indicated that death receptors may be up-regulated by estrogen deprivation, these parameters were evaluated in a series of tumors after pre-operative anti-estrogen therapy. Primary breast tumors from 35 pre-menopausal, progesterone receptor (PR) positive, breast cancer patients were

obtained. Nineteen patients had not received pre-operative treatment; 16 patients had received pre-operative tamoxifen (40 mg p.o., daily for 7-10 days), and LH-RH agonist gosereline (3.6 mg s.c. injection, once). Normal breast samples (n=5) were used as control.It was shown that death receptors DR4 and DR5 were abundantly present immunohistochemically in primary breast tumors of PR+ pre-menopausal patients, while they were mostly absent in normal breast tissue. Short-term anti-estrogen treatment did not further increase this. These results indicate that TRAIL could possibly be a tumor specific treatment for PR+ breast cancer, in the future.

Optimizing breast cancer treatment: future perspectives

In early stage breast cancer, the use of adjuvant systemic therapy has made a major impact on treatment in the last decades. Patients with early stage breast cancer have a relatively good prognosis, and improving the possibility to predict clinical benefit of such treatment in these patients is becoming increasingly important. In this light, the detection of breast cancer at a very early, microscopical, stage remains an attractive approach. Theoretically, patients with microscopic disease are at risk for developing distant metastases, and would benefit from systemic treatment. In breast cancer, like in most solid tumors, no tumor-specific markers are routinely available for detection yet. In trying to find 'the needle in a haystack' this constitutes particular problems with regard to sensitivity and specificity of detection. Quantification of mRNA signals (particularly with real-time methods) has greatly improved molecular detection of micrometastases. However, a large variation may exist in the expression of tumor-associated tissue-specific markers between tumor cells (chapters 3 and 4 of this thesis). Therefore, the distinction between tumor and non-tumor cells by expression levels of tissue specific markers remains difficult. The real-time qRT-PCR of EGP-2 may prove valuable for the evaluation of blood samples. However, real progress in this

field of research should come from discovering truly tumor-specific detection methods, for instance based on tumor specific mutations (such as the p53 tumor-suppressor gene). On the other hand, more traditional immunostaining methods may well prove to be worthy in providing additional staging information. Particularly the aspect of the visual confirmation of the tumor cell is unsurpassed by other detection methods. The labor intensity of this method may be reduced to acceptable proportions by a first automated screening of large numbers of cells (1). Removal of tumor cells, or 'purging' presents an exciting entity in the field of micrometastatic breast cancer. While bone marrow micrometastases may become an additional staging parameter in breast cancer patients without apparent other distant metastases (2), the impact of tumor cells in the PBSC is less clear. A number of large randomized trials are shortly expected to clarify the role of high-dose chemotherapy and PBSC transplantation in the adjuvant breast cancer treatment setting (3). Investigating tumor cell presence in PBSC of patients who participated in those trials, and relating this to clinical follow-up data, may finally allow evaluation of the impact of these tumor cells. If they are found relevant, this would provide a rationale for removing micrometastatic disease from PBSC.

As with PBSC, there is a risk of micrometastatic disease in ovarian tissue of cancer patients. Transplantation of cryopreserved ovarian tissue in female cancer survivors, for maintaining endocrine function and fertility, harbors the possible risk of micrometastases as a source of relapse. While the risk of these micrometastases could be investigated by systematic evaluation of ovarian tissue in (breast) cancer patients, methods for maintaining fertility safely in these women may be further pursued. Besides techniques for purging tumor cells (described in chapter 7), developments regarding maturation of follicles may have an impact. At this moment, it appears that follicles are best matured in the woman, within the ovarian tissue. However, transplanting this tissue harbors the risk of micrometastatic cancer. If it would be

possible to mature selected follicles in-vitro, the risk of micrometastases could presumably be reduced. In-vitro maturation of follicles from young women is not possible at this moment, but it may be in the future.

Breast cancer treatment is likely to become increasingly focussed on optimizing treatment for individual patients. To this end, fundamental research is becoming more and more translated into clinical practice. Tailor-made treatment of breast cancer, by analyzing features of the primary tumor and focussing treatment upon them, may become a realistic option. As described in chapter 10 of this thesis for instance, the DR4 and DR5 receptors on breast tumors, could imply the possibility of potential treatment with their ligand TRAIL. Determination of a genetic expression profile of primary tumors, for instance by means of a selected micro-array system, may allow a quick assessment of the tumors' expected (in)sensitivities for certain treatment modalities, in the future. The increasing insight of the molecular background of tumor growth, metastasizing potential, and treatment sensitivity, together with improving technical facilities, will allow increasingly rational treatment choices in oncology. This development will certainly support the optimization of breast cancer treatment.

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Abbreviations:

PBSC:	peripheral blood stem cells
EGP-2:	epithelial glycoprotein-2
CK19:	cytokeratin-19
HE:	hematoxylin-eosin
qRT-PCR:	quantitative reverse transcriptase-polymerase chain reaction
mRNA:	messenger RNA
BIS-1:	bispecific antibody-1
rhG-CSF:	recombinant human granulocyte-colony stimulating factor
TRAIL:	TNF-related apoptosis inducing ligand

Hoofdstuk 12

Nederlandse samenvatting

Optimaliseren van de behandeling van borstkanker: detectie van micrometastasen

Borstkankerpatiënten zonder aantoonbare uitzaaiingen bij het stellen van de diagnose, kunnen na verloop van tijd toch opnieuw ziekteactiviteit ontwikkelen. Waarschijnlijk waren bij deze patiënten zeer kleine uitzaaiingen, micrometastasen, reeds bij het stellen van de diagnose aanwezig. Gevoelige methoden om micrometastasen te detecteren zouden mogelijk kunnen helpen bij de selectie van patiënten voor vroege systemische ('adjuvante') behandeling. Met name gelet op mogelijke nieuwe behandelingsvormen voor de vroege stadia van borstkanker (zoals bijvoorbeeld hoge-dosis chemotherapie gevolgd door een transplantatie van bloed-stamcellen) wordt een goede selectie van patiënten die hier het meeste baat bij zouden kunnen hebben, belangrijk. Vrouwen voor wie geen gunstig effect van de behandeling valt te verwachten, zouden dan de bijwerkingen van een dergelijke behandeling bespaard kunnen blijven. Door detectie van micrometastasen zou mogelijk tevens het effect van de behandeling beter in kaart kunnen worden gebracht. Ook zou een dergelijke techniek de mogelijkheid bieden het effect van aanwezigheid van micrometastasen in het getransplanteerde bloed-stamcelmateriaal te evalueren, na hoge-dosis chemotherapie. Daarnaast kan de detectie nuttig zijn bij de evaluatie van (in-vitro, d.w.z. celkweek) methoden om micrometastasen op te ruimen.

In hoofdstuk 2 wordt een overzicht gegeven met betrekking tot micrometastasen van borstkanker. Hierbij wordt met name aandacht geschonken aan tumorcellen in bloed-stamcel transplantaten, en de effecten van hematopoïetische groeifactoren. Deze groeifactoren kunnen worden gebruikt bij de behandeling van kanker, om de negatieve effecten van chemotherapie op de bloedaanmaak tegen te gaan. In hoofdstuk 3 wordt het gebruik van de marker

(celkenmerk) 'epitheliaal-glycoproteine-2' (EGP-2) bestudeerd, voor detectie van enkele tumorcellen. EGP-2 komt voor op de celmembraan van alle tumorcellen van epitheliale origine, en zo ook op de celmembraan van borstkankercellen. Als detectiemethoden werden gebruikt: de kwantitatieve 'reverse-transcriptase polymerase chain reaction' (qRT-PCR), alsmede aankleuringen van tumorcellen met antilichamen. Met behulp van de qRT-PCR kan de mRNA expressie van een bepaald gen (de mate waarin het gen wordt 'afgelezen' in de vorm van messenger RNA) worden gemeten. In dit geval is de expressie van EGP-2 specifiek voor epitheliale cellen, maar niet persé voor borstkanker. Voor de opsporing van borstkankercellen in bijvoorbeeld bloed, wordt daarom gebruik gemaakt van het feit dat epitheliale cellen in principe niet in het bloed voorkomen. Naast de marker EGP-2, werd in dit hoofdstuk ook gebruik gemaakt van de epitheliale marker cytokeratine 19. Deze marker wordt vaak gebruikt voor het onderzoek naar micrometastasen. Wij vonden dat de expressie van EGP-2 mRNA per tumor erg wisselde. Het vaststellen van een grens in bloed tussen achtergrond-expressie en expressie die wèl op tumorcellen duidt, wordt door de wisselende expressie in tumoren bemoeilijkt.

In hoofdstuk 4 werd gebruik gemaakt van een serie bloedmonsters (verzameld voor-, tijdens-, en na de operatie), en tumoren van dezelfde borstkankerpatiënten. Tevens kon bij dezelfde patiënten de zogenaamde schildwachtklier worden bestudeerd. Deze lymfklier uit de okselregio, wordt beschouwd als de eerste klier waar de borstkanker zich naar toe uitzaait. In deze studie werd wederom gebruik gemaakt van antilichaamkleuringen. Daarnaast werd de qRT-PCR geautomatiseerd met behulp van de 'real-time' methode. Hiermee worden de expressiesignalen in de loop van de tijd gemeten, in plaats van aan het einde van de qRT-PCR reactie. Met behulp van deze technieken kon zo laag als 1 tumorcel per 1 tot 2 miljoen leukocyten (kernhoudende cellen in het bloed) worden gemeten. In controle lymfklierweefsel van patiënten zonder kanker werd aspecifieke CK19 kleuring gezien, maar geen qRT-PCR

signaal. Ook controle bloedmonsters waren negatief. In de primaire borsttumoren van 58 patiënten werd weer een grote variatie gevonden in de expressie van EGP-2 en CK19. Bij de schildwachtklier van 16 patiënten werden tumorcellen aangetoond met routine morfologisch onderzoek of met antilichaamkleuring voor EGP-2 en CK19. Met de qRT-PCR methode werden echter ook vals-positieve en vals-negatieve uitslagen waargenomen, ondanks dat er een correlatie tussen de aanwezigheid van tumor was met de hoogte van het expressie niveau. De bloedmonsters (149 totaal) waren allemaal negatief met de antilichaamkleuringen, maar met de qRT-PCR hadden 19 patiënten (één derde deel van de patiënten) op één of meerdere momenten een positief monster.

Concluderend werd ook in deze studie een grote variatie van expressie van EGP-2 en CK19 gevonden in primaire borsttumoren. Omdat niet alle tumorcellen deze markers simultaan op een hoog niveau tot expressie brengen, is de sensitiviteit (gevoeligheid) van de detectie waarschijnlijk gebaat bij het gebruik van beide markers, los van elkaar. Bij de schildwachtklieren werd een sensitievere en specifiekere detectie gevonden m.b.v. antilichaamkleuringen dan met de qRT-PCR methode. Een voordeel van de antilichaamkleuringen was, dat door visuele controle een onderscheid tussen aspecifieke kleuring en tumorcellen gemaakt kon worden. De bloedmonsters waren allemaal negatief met antilichaamkleuringen, terwijl juist bij een groot deel van de patiënten een bloedmonster positief werd bevonden met qRT-PCR. Mogelijk geeft dit aan dat de gevoeligheid van de qRT-PCR methode voor de detectie van micrometastasen in bloedmonsters, beter is dan die van de antilichaamkleuringen. De betekenis van deze bevindingen zal duidelijk worden, bij het koppelen met de gegevens over het klinische beloop op lange termijn. Een belangrijke bevinding van deze studie was dat de negatieve controles negatief bleven met de qRT-PCR methode. Hierdoor verviel de noodzaak van het koppelen van

tumorweefsel aan bloed voor de bepaling van een expressie grens voor EGP-2 of CK19, met de vernieuwde real-time qRT-PCR methode.

In hoofdstuk 5 werd derhalve deze qRT-PCR methode toegepast op bloed- en bloed-stamcelmonsters, die waren verzameld van 59 borstkankerpatiënten, vooraf, tijdens en na de behandeling. Deze patiënten, met vier of meer tumor positieve okselklieren, namen allemaal deel aan een landelijke studie waarin het effect van standaard-dosis chemotherapie werd vergeleken met hoge-dosis chemotherapie plus bloed-stamcel transplantatie. Van de 174 monsters werden er met de antilichaamkleuringen tumorcellen aangetroffen bij twee patiënten: in één bloedmonster en één bloed-stamcelmonster. Met de qRT-PCR methode waren monsters van 13 patiënten positief. Het betrof hierbij 8 bloedmonsters en 5 bloed-stamcelmonsters. Eén patiënt had een positief monster met beide technieken, maar wel op verschillende tijdstippen. Ook voor deze studie geldt, dat de klinische betekenis van dit soort bevindingen pas duidelijk kan worden, indien gecombineerd met gegevens over het klinische beloop van een grote groep van dergelijke patiënten.

Optimaliseren van de behandeling van borstkanker: verwijderen van micrometastasen

In hoofdstuk 6 werd een methode beschreven om in een experimenteel in-vitro systeem micrometastasen te kunnen verwijderen vanuit bloed-stamcellen. De achtergrond hiervoor is, dat de aanwezigheid van micrometastasen in bloed-stamcelmateriaal, na transplantatie mogelijk aanleiding kan geven tot terugkeer van de ziekte. Epitheliale tumorcellen kunnen specifiek worden opgeruimd door gebruik te maken van bispecifiek antilichaam BIS-1. Dit antilichaam is gericht tegen EGP-2 en de T cel receptor CD3. Door de verbinding die het bispecifieke antilichaam kan leggen tussen de tumorcellen en geactiveerde T cellen (die een belangrijke rol spelen

bij de afweer van het lichaam), kunnen de tumorcellen zeer specifiek door de T cellen worden gedood. De T cellen, die aanwezig zijn in het bloed-stamcelmateriaal, werden hiervoor gebruikt. Bij het bestuderen van de samenstelling van het bloed-stamcelmateriaal werd al gevonden dat dit materiaal meer cytotoxische T cellen bevat dan normaal controle bloed. Na verdere activatie van deze T cellen, m.b.v. het antilichaam gericht tegen de CD3 receptor, werd een toename gezien van de tumorceldood door de T cellen, die nog verder toenam o.i.v. BIS-1. Het maximale effect van het toevoegen van BIS-1 werd na 72 uur pre-activatie gezien, waarbij een > 3 log tumorceldood werd gevonden. Dit betekent dat meer dan 99,9% van de tumorcellen werden gedood door deze methode. Een belangrijk aspect hierbij was dat het delingspotentieel van de bloed-stamcellen door deze procedure niet werd aangetast. Daarom werd geconcludeerd dat in een in-vitro model specifieke tumorceldood door bloed-stamcelmateriaal zelf, kan worden bereikt door pre-activatie van dit materiaal, gevolgd door BIS-1 toediening.

Op basis van deze resultaten werd deze methode toegepast in een andere situatie, beschreven in hoofdstuk 7. Hierbij werd een experimenteel in-vitro model ontwikkeld om enkele epitheliale tumorcellen te verwijderen uit ovarium (eierstok)weefsel. Door chemotherapie of radiotherapie kunnen de eierstokken van jonge vrouwen dermate aangetast raken, dat ze er onvruchtbaar door worden. Het invriezen van eierstokweefsel, om het na de behandeling terug te plaatsen bij de vrouw zelf, is een potentiële nieuwe methode om de fertiliteit (vruchtbaarheid) van deze vrouwen te handhaven. Echter, het risico bestaat dat micrometastasen in het eierstokweefsel aanleiding kan geven tot terugkeer van de ziekte. Daarom werd in-vitro het effect van geactiveerde T cellen en BIS-1 op tumorceldood bestudeerd, in de aan- of afwezigheid van eierstokweefsel. In dit systeem werd geen negatief effect gezien van het toevoegen van eierstokweefsel op tumorceldood, veroorzaakt door de T cellen met BIS-1. Een belangrijke bevinding was dat het eierstokweefsel na de

procedure nog steeds morfologisch intacte eicellen bevatte. Mogelijkerwijs kan deze methode bijdragen aan het veilig terugplaatsen in de toekomst van eierstokweefsel bij jonge vrouwen, na behandeling zijn voor kanker.

Meer manieren om de behandeling van borstkanker te optimaliseren

In hoofdstuk 8 werd de hematopoïetische groeifactor rhG-CSF (recombinant human granulocyte-colony stimulating factor) gebruikt voor de preventie van koorts bij leucopenie. Bij leucopenie is het aantal witte bloedcellen dat voor de afweer zorgt sterk gedaald; dit komt (tijdelijk) voor door chemokuren. Omdat de afweer hierdoor verminderd is, kan koorts ontstaan. Door het toedienen van rhG-CSF kan de vermindering van het aantal cellen voor de afweer door de chemokuur worden tegengegaan. In een gerandomiseerde studie bij 40 borstkankerpatiënten, die intermediaire hoge-dosis chemotherapie kregen (cyclophosphamide, 5-fluorouracil plus epirubicine of methotrexaat), werd het gebruik van preventieve rhG-CSF vergeleken met preventieve antibiotica (ciprofloxacine en amfotericine B). In de groep die rhG-CSF kreeg, ontwikkelden 7 van de 18 patiënten koorts bij leucopenie (na 10 van de 108 kuren); in de groep die antibiotica kreeg, gebeurde dit bij 7 van de 22 patiënten (na 7 van de 98 kuren); er zat geen verschil tussen beide groepen. De duur van de ziekenhuisopname en de kosten hiervan waren ook niet verschillend voor beide groepen, maar rhG-CSF gebruik was wel 6,6 keer duurder per kuur dan de preventieve antibiotica. De conclusie was, dat ciprofloxacine en amfotericine B een vergelijkbare effectiviteit hebben als rhG-CSF ter voorkoming van koorts bij leucopenie, en goedkoper zijn.

In hoofdstuk 9 werd onderzocht of bloed-stamcellen verouderen door hoge-dosis chemotherapie en transplantatie van bloed-stamcellen. Veroudering van bloed-stamcellen kan mogelijk ongunstige gevolgen hebben op de lange termijn, zoals

maligne afwijkingen in de bloedvormende cellen. Juist bij patiënten met een goede prognose, zijn dat soort lange termijn effecten klinisch relevant. Door het meten van telomeerlengte kan iets worden gezegd over de leeftijd van cellen, omdat telomeren (de uiteinden van chromosomen) bij elke normale celdeling iets korter worden. Telomerase is het enzym dat deze verkorting kan tegengaan. Bij borstkankerpatiënten, die adjuvante behandeling kregen met standaard-dosis chemotherapie (17 patiënten), of hoge-dosis chemotherapie en transplantatie van bloed-stamcellen (16 patiënten), werd daarom telomeerlengte en telomerase activiteit gemeten, voor en na de behandeling. Ook werden de waarden in het bloed gemeten van hemoglobine, MCV, leukocyten en thrombocyten, voorafgaand aan de chemotherapie, en 5 en 9 maanden daarna (t_0, t_1, en t_2). Deze waarden in het bloed waren verlaagd op t_1 en t_2 vergeleken met t_0 (hoge-dosis groep: alle waarden; standaard-dosis groep: leukocyten en thrombocyten), en alle waarden waren lager na hoge-dosis chemotherapie vergeleken met standaard-dosis chemotherapie op t_1. Gepaarde leukocytenmonsters van t_0 en t_1 lieten telomeerlengte veranderingen zien van +0.8 tot –2.2 kb, waarbij 9 patiënten in beide groepen een verkorting hadden (deze verandering was niet significant). Telomerase activiteit bleef onder de detectiegrens in leukocytenmonsters op t_0 en t_1. De conclusie was, dat zowel standaard- als hoge-dosis chemotherapie een negatief effect op de waarden in het bloed (hemoglobine, MCV, leukocyten en thrombocyten) hebben. Ondanks het feit dat de bloed-stamcellen in deze situatie waarschijnlijk sneller hebben moeten delen om dit negatieve effect te compenseren, werd slechts bij een deel van de patiënten een verkorting van telomeerlengte gevonden, die niet aan de chemotherapie dosering was gerelateerd.

In hoofdstuk 10 werden primaire borsttumoren bestudeerd. Apoptose, d.w.z. gereguleerde celdood, kan in tumorcellen o.a. worden veroorzaakt door middel van de Tumor Necrosis Factor (TNF) familie van receptoren. Deze familie bestaat o.a. uit de zogenaamde 'death receptors' Fas (de receptor voor Fas Ligand, FasL) en DR4 en DR5

(receptoren voor 'TNF-Related Apoptosis Inducing Ligand, TRAIL). Behandeling met TRAIL zou mogelijk effect kunnen hebben bij tumoren met DR4 en DR5 receptoren. Daarom werd de aanwezigheid van Fas, FasL, DR4 en DR5 in borsttumoren bestudeerd, met behulp van antilichaamkleuringen. Verder werd tumorcelproliferatie, celdood en inhibitie van celdood bestudeerd. Omdat uit in-vitro studies is gebleken dat het onttrekken van oestrogenen aan borstkankercellen effect kan hebben op deze parameters, werd hier ook naar gekeken in een groep primaire borsttumoren, na anti-oestrogene voorbehandeling. Primaire borsttumoren van 35 pre-menopauzale hormoonreceptor-positieve borstkankerpatiënten werden bestudeerd. Negentien patiënten waren niet voorbehandeld; 16 patiënten hadden voorafgaand aan de operatie dagelijks 40 mg tamoxifen (een oestrogeen antagonist) gedurende 7-10 dagen gekregen, plus een injectie van 3,6 mg gosereline (een agonist van LH-RH: luteinizing hormone releasing hormone). De combinatie van deze medicijnen doet de oestrogeen spiegel bij pre-menopauzale vrouwen dalen, tot een niveau dat vergelijkbaar is met de situatie na verwijdering van de eierstokken. De 'death receptors' DR4 en DR5 waren ruimschoots aanwezig in de meerderheid van de borsttumoren, terwijl dit bij normaal borstweefsel niet- of veel minder het geval was. Anti-oestrogene behandeling voorafgaand aan de operatie beïnvloedde deze aanwezigheid verder niet. Deze resultaten geven aan dat TRAIL mogelijk een tumor-specifieke behandeling voor hormoonreceptor-positieve borstkanker van pre-menopauzale patiënten zou kunnen vormen in de toekomst.

Optimaliseren van de behandeling van borstkanker: toekomstperspectieven

Bij de vroege stadia van borstkanker heeft de invoering van adjuvante systeemtherapie een groot effect op de behandeling gehad. Patiënten met borstkanker in een vroeg stadium hebben een relatief gunstige prognose. Daarom is het van belang

om de klinische voordelen voor individuele patiënten goed te kunnen inschatten van tevoren. In dat kader is het aantrekkelijk om borstkanker in een zeer vroeg, microscopisch stadium te kunnen detecteren. Theoretisch lopen patiënten met micrometastasen risico op het ontwikkelen van uitzaaiingen, en zouden zij bij uitstek baat kunnen hebben bij vroegtijdige systemische behandeling. Net als bij de meeste andere zogenaamde solide tumoren, is er bij borstkanker nog geen gangbare tumor-specifieke marker beschikbaar voor de detectie van micrometastasen. Bij het zoeken naar de 'speld in de hooiberg', brengt dit moeilijkheden met zich mee met betrekking tot de sensitiviteit en specificiteit van de detectie. Het kwantificeren van de expressie van de beschikbare tumor geassociëerde weefsel-specifieke markers (met name met de geavanceerde metingen continue in het verloop van de tijd), heeft de moleculaire detectie van micrometastasen zeer verbeterd. Mogelijk kan deze detectiemethode van waarde zijn voor de evaluatie van bloedmonsters (hoofdstuk 4 en 5 van dit proefschrift). In de expressie van deze weefel-specifieke markers in primaire tumoren wordt echter een grote variatie aangetroffen (hoofdstuk 3 en 4 van dit proefschrift). Daardoor blijft het maken van onderscheid tussen tumor en gewone cel moeilijk. Werkelijke vooruitgang in dit onderzoeksgebied is dan ook met name te verwachten, als echt tumor-specifieke markers worden ontdekt. Dit zouden bijvoorbeeld tumor-specifieke mutaties kunnen zijn (zoals mutaties in het p53 tumor-suppressor gen). Aan de andere kant blijken de traditionelere antilichaamkleuringen steeds vaker van waarde te zijn. De mogelijkheid om visueel te controleren of de aangekleurde cel werkelijk een tumorcel is, is een voordeel dat andere detectiemethoden nu niet bieden. Het feit dat deze methode arbeidsintensief is, kan mogelijk worden opgevangen door het automatiseren van de eerste evaluatie op hoofdlijnen van grote aantallen cellen (1), waarna het fijnere onderzoek van daadwerkelijk aangekleurde cellen sneller kan worden afgerond.

Terwijl micrometastasen in het beenmerg mogelijk een aanvullende stageringsparameter kan worden bij borstkankerpatiënten zonder duidelijke uitzaaiingen op afstand (2), is de rol van micrometastasen in bloed-stamcelmateriaal minder duidelijk. De resultaten van een aantal grote, gerandomiseerde studies zullen binnenkort duidelijkheid geven over de rol van hoge-dosis chemotherapie en bloed-stamcel transplantaties bij de vroege, adjuvante behandeling van borstkanker (3). Het bestuderen van eventuele aanwezigheid van micrometastasen in bloed-stamcelmateriaal van patiënten die in het kader van deze studies zijn behandeld, en het relateren aan het klinische beloop in de tijd, zal uiteindelijk duidelijkheid geven over het effect van micrometastasen in bloed-stamcelmateriaal. Dit kan dan de rationele onderbouwing bieden voor het al dan niet verwijderen van deze micrometastasen.

Zoals bij bloed-stamcelmateriaal, bestaat ook bij eierstokweefsel van kankerpatiënten de kans op micrometastasen. Voor het mogelijke behoud van vruchtbaarheid bij jonge vrouwen die voor kanker behandeld worden, is een methode in ontwikkeling waarbij eierstokweefsel van deze vrouwen kan worden ingevroren. Het doel hierbij is om het weefsel t.z.t., na de behandeling voor kanker, bij hen terug te plaatsen. Terugplaatsen van eierstokweefsel zou echter, bij de aanwezigheid van micrometastasen, het risico op terugkeer van de ziekte met zich mee kunnen brengen. Terwijl de kans op de aanwezigheid van deze micrometastasen verder onderzocht zou kunnen worden door eierstokweefsel van (borst)kankerpatiënten systematisch hierop te evalueren, kunnen de methoden voor het veilige behoud van vruchtbaarheid bij deze vrouwen verder worden uitgebouwd. Naast de technieken om de tumorcellen uit eierstokweefsel te verwijderen (zoals beschreven in hoofdstuk 7), kunnen hierbij ook ontwikkelingen op het gebied van het uitrijpen van eicellen van belang zijn. Op dit moment lijkt het dat eicellen het beste in de vrouw kunnen uitrijpen, in het ondersteunende eierstokweefsel. Indien echter geselecteerde eicellen in een

kweeksysteem zouden kunnen uitrijpen (in vitro maturatie), zal daardoor het risico van micrometastasen niet meer aanwezig zijn. Een dergelijke kweekmethode voor eicellen van (jonge) vrouwen is op dit moment echter nog niet goed mogelijk.

De behandeling van borstkanker is in toenemende mate gericht op het optimaliseren van de behandeling van individuele patiënten. Hiertoe worden onderzoeksgegevens steeds meer vertaald naar de klinische praktijk. Behandeling van borstkanker 'op maat', door het in kaart brengen van de eigenschappen van de primaire tumor en het toespitsen van de behandeling hierop, lijkt geen utopie meer. Zoals bijvoorbeeld in hoofdstuk 10 van dit proefschrift werd besproken, zou de aanwezigheid van de receptoren DR4 en DR5 in borsttumoren wellicht iets kunnen zeggen over de mogelijkheid van behandeling met hun 'ligand' TRAIL, of een modificatie hiervan. Een snelle, klinisch toepasbare, evaluatie van de verwachte (on)gevoeligheid van primaire tumoren voor verschillende behandelingen kan in de toekomst wellicht een reële optie worden, door de vele (genetische) eigenschappen van tumoren te onderzoeken met nieuwe technieken (zoals een selectief micro-array systeem). De toename van inzicht in de moleculaire achtergrond van tumorgroei, uitzaaiingen en gevoeligheid voor behandelingen, tezamen met de verbetering van beschikbare technieken, biedt de mogelijkheid van steeds beter gefundeerde keuzen in de behandeling. Deze ontwikkeling zal de optimalisatie van de behandeling van borstkanker verder kunnen bevorderen.

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Lab H-0, en iedere bewoner: er komt vast een tijd dat we met nostalgie aan de oudbouw kunnen terugdenken!

Paranimfen Gerda en Lydia: in de afgelopen jaren hebben we al vele belangrijke gebeurtenissen in ons leven met elkaar kunnen delen, en ik ben blij dat jullie er ook op 2 juli bij zijn.

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Dan tot slot: Edmond en Pim. Laat ik kort zijn. Heren, wat zou er aan zijn zonder jullie? Precies.

Carolien