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New ways to optimize breast cancer treatment

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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

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