Modulation of death receptor-mediated apoptosis in cervical neoplasia
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Cervical cancer is the most frequent occurring gynaecologic malignancy world-wide. Approximately 492,000 women develop cervical cancer and 274,000 die from it each year (global estimate for 2002). Less developed countries display the highest cervical cancer incidence rates due to lack of effective screening programs. Sexually transmitted infection with high-risk types of the human papillomavirus (HPV), mainly HPV16 and HPV18, is the primary risk factor for the development of cervical cancer and its precursor lesions. These precursor lesions, or cervical intraepithelial neoplasia (CIN), are subdivided in low (CIN I), moderate (CIN II) or high (CIN III) grade lesions. Most CIN I lesions will regress spontaneously, while 20-45% of untreated CIN II/III lesions will persist and eventually progress to cervical cancer when left untreated. Current optimal therapy of locally advanced cervical cancer consists of radiation in combination with cisplatin-based chemotherapy. However, the 5-year overall survival is still only 52%. To improve treatment results, attention is focused on the discovery of innovative therapeutic strategies. Drugs directed at inducing tumour cell apoptosis are currently envisioned as interesting cancer treatment modalities.

Important apoptosis inducers are members of the tumour necrosis factor (TNF)-receptor family and their cognate ligands. Among the well characterised death ligands are Fas Ligand (Fasl) and TNF-related apoptosis-inducing ligand (TRAIL), which induce apoptosis in susceptible cells expressing the cell surface death receptors Fas and DR4/DR5, respectively. Ligand binding to the receptor triggers the formation of a death-inducing signalling complex (DISC), which consists of a trimerised receptor complex, the adaptor protein FADD and caspase 8. Caspase 8 is activated at the DISC level and activates other downstream executioner caspases. In response to this activated caspase cascade, specific death substrates are cleaved resulting in cellular disassembly characteristic for apoptosis. The apoptotic pathway can be inhibited at multiple stages in the signalling cascade by increased expression of inhibitory proteins such as cFLIP, the inhibitor of apoptosis proteins (IAPs) and the anti-apoptotic members of the Bcl-2 protein family.
The apoptotic pathway is very complex and involves many proteins with pro- or anti-apoptotic functions. Differential expression of these proteins can result in resistance to apoptosis.

In chapter 2 an overview is presented of the molecular options to change the apoptotic balance in cervical cancer through enhanced death receptor-mediated apoptosis, proteasome inhibitors, short interfering RNAs (siRNA) and non-steroidal anti-inflammatory drugs (NSAIDs). In addition, the potential of attacking pro-survival signalling via the epidermal growth factor receptor (EGFR) and insulin-like-growth factor receptor (IGF-R) to support the apoptotic process is discussed. Combining innovative therapeutic agents with standard treatment such as chemo- and radiotherapy may induce apoptosis more potently compared to single agent treatment. The emerging scenario for treatment of cervical cancer probably requires the rational combination of death ligands plus conventional therapy such as radiotherapy and "classic" cytostatic drugs in regimens that optimise anti-neoplastic activity towards malignant cells. This may be achieved through restoration of the apoptotic pathway thus bypassing chemo- or radiotherapy resistance of malignant cells. Further translational and clinical research is needed to elucidate the potential of these compounds for the treatment of cervical cancer.

Binding of Fas ligand or apoptosis-inducing anti-Fas antibody (anti-Fas) to the death receptor Fas can activate a caspase-cascade resulting in apoptosis. In chapter 3 the functionality of the Fas pathway was studied in human cervical cancer cells with different HPV and p53 status. HeLa (HPV-18 positive), CaSki and SiHa (both HPV-16 positive) contain wild-type p53, while C33A (HPV negative) expresses mutant p53. Despite high Fas membrane expression in all HPV-positive cells, CaSki was highly sensitive, HeLa slightly sensitive, while SiHa and C33A were resistant to anti-Fas. Almost undetectable Fas membrane levels can explain the non-responsiveness of C33A for anti-Fas. Although interferon-γ (IFNγ) strongly and cisplatin to a lesser extent enhanced Fas membrane expression in all HPV-positive cells, sensitisation to anti-Fas by IFNγ or cisplatin was only observed in HeLa. Analysis of the Fas apoptotic pathway showed that anti-
Many proteins with diverse functions are targets of these proteins can be silenced using short interfering RNAs (siRNA) or shRNAs. In addition, the use of irradiation or dermal growth factor receptor (IGF-R) to support innovative therapeutic strategies and radiotherapy may be an alternative agent treatment. The success of this approach probably requires the development of combinational therapy such as chemotherapies that optimise antitumor effects. This can be achieved through radiosensitisation, chemotherapy and clinical research is ongoing to improve strategies for the treatment of cervical cancer.

The Fas/FasL system leads to a caspase cascade resulting in cell death. In chapter 3, the Fas/FasL cascade pathway was studied with respect to Fas, caspase-8, caspase-9 and p53 status. HeLa (sensitive) and CaSki (resistant) contain wild-type and mutant p53. Despite high levels of Fas, CaSki was highly resistant to Fas treatment, which may explain the non-sensitivity to Fas. Interferon-γ (IFNγ) strongly induced Fas expression in all cervical cancer cell lines, with cisplatin only slightly increasing Fas expression. The Fas treatment induced caspase-8 activation and concomitantly Bid cleavage, caspase-9 and caspase-3 activation, PARP cleavage and apoptosis in HeLa and CaSki. IFNγ plus anti-Fas treatment, in contrast to anti-Fas alone, facilitated caspase-8 activation in HeLa and SiHa, while an increase in Bid cleavage, caspase-9 activation and apoptosis was only observed in HeLa. Apoptotic failure in SiHa (even in the presence of IFNγ) was probably due to low caspase-8, almost undetectable Bid protein levels and therefore lack of caspase-9 activation. It was concluded that sensitivity to anti-Fas depends on Fas, caspase-8 and Bid protein levels in cervical cancer cells. Additionally, IFNγ and cisplatin can increase sensitivity to anti-Fas in a subset of HPV-positive cervical cancer cell lines by upregulation of Fas and caspase-8 expression without major changes in p53 levels.

In cervical carcinogenesis the p53 and pRb tumour suppressor pathways are disrupted by HPV E6 and E7 oncogene expression, respectively. E6 targets p53 and E7 targets pRb for rapid proteasome-mediated degradation. In chapter 4 we investigated whether proteasome inhibition by MG132 could restore wild-type p53 levels and sensitise HPV-positive cervical cancer cell lines to apoptotic stimuli such as rhTRAIL. Similar to anti-Fas (chapter 3), CaSki was highly sensitive, HeLa intermediate and SiHa not sensitive to recombinant human TRAIL (rhTRAIL)-induced apoptosis. MG132 strongly sensitised HeLa and SiHa to rhTRAIL-induced apoptosis in a caspase- and time-dependent manner. MG132 massively induced TRAIL receptor DR4 and DR5 membrane expression in HeLa, whereas in SiHa only DR5 membrane expression was upregulated from almost undetectable to high levels. Antagonistic DR4 antibody partially inhibited apoptosis induction by rhTRAIL and MG132 in HeLa but had no effect on apoptosis in SiHa. Inhibition of E6-mediated p53 proteasomal degradation by MG132 resulted in elevated levels of active p53 as was demonstrated by p53 siRNA sensitive p21 upregulation. Although p53 siRNA partially inhibited MG132-induced DR5 upregulation in HeLa and SiHa, no effect on rhTRAIL-induced apoptosis was observed. MG132 plus rhTRAIL enhanced caspase 8 and caspase 3 activation and concomitant cleavage of XIAP, particularly in HeLa. In addition, caspase 9 activation was only observed in HeLa. Downregulation of XIAP using small interfering RNA (siRNA)
in combination with rhTRAIL induced high levels of apoptosis in HeLa, whereas MG132 had to be added to the combination of XIAP siRNA plus rhTRAIL to induce apoptosis in SiHa. In conclusion, proteasome inhibition sensitised HPV-positive cervical cancer cell lines to rhTRAIL independent of p53. Our results indicate that DR4 and DR5 upregulation but also XIAP inactivation contribute to rhTRAIL sensitisation by MG132 in cervical cancer cell lines. Combining proteasome inhibitors with rhTRAIL may be therapeutically useful in cervical cancer treatment.

In chapter 5 we investigated whether E6 and/or E7 suppression by RNAi, using siRNA, restores p53 and pRb functionality and sensitises the resistant HPV16-positive cervical cancer cell line SiHa to apoptosis. Treatment with E6 siRNA resulted in enhanced p53 expression, upregulation of p21 and cell growth inhibition. Enhanced p53 expression and cell growth inhibition were observed with E7 siRNA without any effect on pRb expression levels. E6 and/or E7 suppression did not induce high levels of apoptosis or confer susceptibility to rhTRAIL, anti-Fas or irradiation. In contrast, cisplatin induced a considerable percentage of apoptosis in E6 suppressed cells. Cisplatin treatment had no effect on p53 levels in these cells but resulted in down regulation of p21 and enhanced activation of caspase 8. Moreover, cisplatin sensitised especially E6 suppressed cells to rhTRAIL or anti-Fas-induced apoptosis. Following treatment with cisplatin in combination with death ligands, increased caspase 8 as well as caspase 3 activation was observed in E6 suppressed cells. This effect was independent of upregulation of death receptor membrane expression. In conclusion, E6 siRNA in combination with cisplatin can efficiently potentiate rhTRAIL or anti-Fas induced apoptosis due to an enhanced activation of caspase 8.

Increasing imbalance between proliferation and apoptosis (in favour of proliferation) is important in cervical carcinogenesis. The death ligands FasL and TRAIL induce apoptosis by binding to their cognate cell-surface death receptors Fas or death receptor (DR) 4 and DR5. In chapter 6 we examined if changes in death ligand and death receptor expression at different stages of cervical carcinogenesis are related to an imbalance between proliferation and apoptosis. The immunohistochemical expression and localisation of
apoptosis in HeLa,
expression of XIAP siRNA plus MG132 sensitises the resistant cells to apoptosis. Treatment with RNAi downregulation of p21 and p27, cell growth inhibition and p21 expression levels. XIAP inhibition apoptosis or confer resistance to apoptosis. In contrast, cisplatin suppressed cells, caspase 8. Moreover, rhTRAIL or anti-Fas-Ligand in combination with MG132 sensitises caspase 3 activation was independent of caspase 8. In conclusion, E6 potentiate rhTRAIL or MG132 sensitisation of caspase 8.

In this study, we examined apoptosis (in favour of cell death), cell-surface death ligands FasL and DR4/DR5/Trail, and localisation of Fas/FasL and DR4/DR5/Trail were assessed in 11 normal cervixes, 15 CIN I, 15 CIN II, 13 CIN III and 25 (microinvasive) squamous cell cervical cancers. A marked increase in proliferation as well as apoptosis percentage was found with increasing severity of neoplasia. In normal cervix and CIN I samples FasL, DR4, DR5 and TRAIL staining was mainly observed in the basal/parabasal layer, whereas Fas staining was localised in the superficial, more differentiated epithelial layer. Frequency of Fas positive staining decreased with increasing severity of CIN. In contrast, homogeneous FasL, DR4, DR5 and TRAIL expression throughout the lesions was more frequently observed in CIN III and cervical cancer. FasL, DR4, DR5 and TRAIL staining patterns were correlated, although TRAIL expression was more intense in low-grade lesions. No association was found between death receptor or ligand expression with the percentage of apoptosis or proliferation. The loss of Fas and the deregulation of FasL, DR4, DR5 and TRAIL in the CIN-cervical cancer sequence suggests a possible functional role of these death ligands and receptors during cervical carcinogenesis. The frequent expression of DR4 and DR5 presents these receptors as promising targets for innovative therapy modalities in cervical neoplasia. However, no data are available about the sensitivity of cervical neoplastic lesions to TRAIL. Preclinical studies revealed that proteasome inhibition by MG132 sensitised cervical cancer cell lines to rhTRAIL (chapter 4). In chapter 7 we therefore evaluated the apoptosis-inducing effect of rhTRAIL and proteasome inhibition by MG132 either as monotherapy or as combination therapy in cervical explants obtained from healthy controls and patients with high-grade cervical intraepithelial neoplasia (CIN III). We established a short-term culture system for ex vivo explants, in which cervical explants from normal cervix and CIN III lesions were exposed to either rhTRAIL, MG132 or their combination and were compared to untreated cultured explants at the level of apoptosis induction. CIN III explants were highly sensitive to MG132 plus rhTRAIL (mean % apoptosis: 91±5) compared to normal cervix treated with MG132 plus rhTRAIL (mean % apoptosis: 24±10, p< 0.0001), while monotherapy with either rhTRAIL, MG132 or medium only resulted in a mean % apoptosis of less than 10 in both CIN III lesions and normal cervix. In conclusion, our study points to a strong synergistic apoptosis-inducing effect of the combination of rhTRAIL and MG132, especially in cell lines.
CIN III lesions. rhTRAIL combined with proteasome inhibitors deserves further exploration as topically applied medical treatment for high-grade premalignant cervical neoplasia.

**DISCUSSION AND FUTURE PERSPECTIVES**

Countries that have implemented organised cervical screening programmes based on the cervical smear, also called Pap cytology test, achieved a reduction in cervical cancer incidence by ≥80%. However, the decrease in cervical cancer in these developed countries has levelled off, indicating that the screening limits may have been reached. In addition, the most critical limitations of the Pap test are its high false-negative and false-positive rate. However, no other diagnostic tools are available that can cost-effectively replace or augment the Pap test. Moreover the Pap test as a cervical screening technology is not widely available in less developed countries where cervical cancer incidence and mortality are high. Cervical cancer thus remains a significant public health concern worldwide. An important question is whether the introduction of new technologies can yield further improvement in the battle against this disease. The knowledge that cervical cancer is associated with infection with high-risk HPV types has led to new research paradigms in the detection and prevention of CIN and cervical cancer. A new screening tool that may serve as an adjunct to the Pap test is HPV DNA detection. The presence of high-risk HPV DNA (even in the presence of a negative Pap test) identifies both women with disease as well as those who are at particular risk of progressing to disease, thereby improving the accuracy of early detection. HPV DNA testing after conservative surgical treatment for CIN may also be helpful in detecting early residual and recurrent disease 9. Additionally, HPV DNA testing is useful as a secondary test (also called triage) for women with minimal cytological abnormalities 10.

Research on HPV has also provided substantial technological knowledge that is currently being used to develop anti-viral vaccination strategies to prevent or treat cervical cancer 11-14. Both preventative as well as therapeutic vaccines have been or are currently being tested in clinical trials. The prospect