The role of cytokines in cancer. With emphasis on the regulation of interleukin-6 expression in human ovarian carcinoma cells.
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Summary and conclusions

Alterations in the expression of and response to growth factors is one of the biological characteristics of tumors. In chapter 1, a review is presented regarding the reported relations between p53 and growth factor regulation. The function of p53 tumor suppressor protein is determined by various intrinsic properties of the protein. The effect of p53 DNA-binding, and protein-protein interactions are determined by the conformation of the protein. Thus, p53 fulfils its role in cell cycle control and the onset of apoptotic cell death, which can be altered when the wild-type p53 (wt-p53) conformation is changed after mutation.

The review focuses on the communal interactions of wt- and mutant p53 (m-p53) with growth factors and shows that m-p53 affects different cell biological functions that determine the malignant behaviour of cells. P53, for instance, can affect the response of cells to growth factors and growth factor-withdrawal. For interleukin-6 (IL-6) signalling it has only recently become clear that p53 is able to inhibit the binding of STAT3 to DNA (1). In this way mutant p53 could abrogate the normally IL-6-induced differentiation of cells and instead promote proliferation. This hypothesis was supported by the results of Bellido et al. (2) who showed that STAT3 protein are involved in the IL-6 induced activation of the p21 (WAF1, CIP1) promoter. P21 plays a crucial role in cell differentiation and especially STAT3 binding seems to be of importance.

Furthermore, p53 is involved in the expression of several growth factor- and growth factor receptor genes. These data suggest that restoration of the wild-type p53 phenotype in tumor cells can not only affect cell cycle control and apoptotic mechanisms but also can reduce autocrine growth and restore sensitivity to physiological growth inhibitors.

Autocrine production of cytokines is one of the biological features of human ovarian carcinomas. Studies with cultured human ovarian tumor-derived cells and cell lines have elucidated some of the aspects of these autocrine growth mechanisms. In the chapters 2 and 4 of this thesis the expression of especially IL-6 in this tumor type is described.

Human ovarian tumors have in over 50 % of the cases a mutated p53 gene. Since in vitro studies have shown a possible regulatory function of p53 protein in the expression of growth factors such as IL-6, we questioned whether the expression of certain growth factors in human ovarian tumors was associated with m-p53. In chapter 2, the relation between p53 expression and cytokine expression is studied in human ovarian tumors. In this study 30 tumors, obtained at diagnosis, are characterized for p53 protein expression with immunohistochemistry techniques and analyzed for the expression of macrophage colony-stimulating factor (M-CSF), IL-6, IL-1β, IL-11, and tumor necrosis factor-α (TNF-α) with Northern blotting. In accordance to earlier studies, nuclear as well as cytoplasmatic p53 staining was observed in 27% of the tumors, whereas 30% showed only cytoplasmatic staining, and 43 % showed no p53 staining. In 70% of the cases M-CSF mRNA was expressed, in 40% TNF-α, and in 30% IL-6. None of the tumors expressed IL-1β or IL-11. The expression of TNF-α occurred more frequently in M-CSF positive tumors compared to M-CSF negative ones. M-CSF expression was associated with nuclear p53 staining. The p53 positive tumors expressed more frequency one or more cytokines compared to p53 negative tumors. TNF-α expression was also associated with response to chemotherapy.

This relatively small study suggests that mutations in the p53 gene might be associated with cytokine overexpression in human ovarian carcinomas, especially the expression of M-CSF mRNA.

Since autocrine production of IL-6 is an important feature of human ovarian carcinomas,
in chapter 3 and 4, the intracellular regulation of IL-6 gene expression in human ovarian tumor cell lines was further investigated. Until now, mechanisms of IL-6 expression and regulation have been analyzed especially in haematopoietic cells and fibroblasts. Therefore, two human ovarian cancer cell lines, the A2780 and the CAOV-3, were analyzed regarding transcription factor DNA-binding status and IL-6 promoter activity. The cells were also exposed to TNF-α, since this factor was shown to be a potent enhancer of IL-6 expression in various cell types.

CAOV-3 cells spontaneously secreted IL-6, which was enhanced by TNF-α. The basic IL-6 expression was associated with activator protein-1 (AP-1), and NF-IL6 DNA binding as determined with electrophoretic mobility shift assay (EMSA). TNF-α also induced nuclear factor kappa-B (NF-κB) which mainly consisted of p65-NF-κB. A2780 cells did neither express IL-6 spontaneously nor after stimulation with TNF-α. EMSAs, however, showed AP-1 DNA binding. TNF-α stimulation enhanced AP-1 and induced NF-κB DNA binding in these cells. No NF-IL6 DNA binding was observed spontaneously or after TNF-α treatment. NF-IL6 protein, however, was detected in nuclear extracts of these cells. Co-stimulation with interferon-γ, a factor which in combination TNF-α induced IL-6 expression in hematopoietic tumor cell lines, induced IL-6 expression in A2780 cells which was associated with increased NF-κB binding but not NF-IL6. This suggests that NF-IL6 DNA binding is not necessary in IL-6 expression in this cell line and that other pathways that also activate NF-κB are involved. IL-6 promoter transfection studies showed no difference in promoter activation between CAOV-3 and A2780 that could explain the fact that A2780 cells do not express IL-6 after stimulation with TNF-α. In addition, no mutations in the endogenous IL-6 enhancer of the A2780 cells were found. This study reveals that differential IL-6 gene expression in two human ovarian cancer cell lines is not dependent on NF-IL6 activation. The signalling pathways induced by TNF-α and/or IFN-γ are probably variable in these cells. NF-κB was most clearly activated in both cell lines after TNF-α and TNF-α/IFN-γ stimulation.

Several studies have indicated a role for p53 in the regulation of IL-6 expression. Therefore, in chapter 4, the effect of p53 phenotypes that frequently occur in human ovarian tumors, on the IL-6 promoter activation, was assessed. The human ovarian wt-p53 cell line A2780 was stably transfected with an empty plasmid (CMV) as control, or plasmids expressing the mutant p53 forms, (m)-175-, m-248-, or m-273-p53. EMSAs revealed a difference in AP-1 DNA-binding activity in the various clones. No differences in NF-κB and NF-IL6 were found. This is somewhat unexpected since earlier studies revealed a role for NF-IL6 in the p53-affected IL-6 activation. Supershift analysis demonstrated that the AP-1/DNA complexes in the various clones had variable compositions. Fra proteins were only present in AP-1 of the m-175 and m-248 clones. Since differences in AP-1 composition could affect AP-1 DNA binding as well as transcriptional activity, the IL-6 promoter activity was evaluated using intact and AP-1 binding site-deleted constructs. Removal of the AP-1 site resulted in a decreased IL-6 promoter activity in the CMV and m-273, and an increased activity in the m-175 clone, while no change was observed in the m-248 clone. Exposure of the p53 clones to TNF-α altered distinctly the AP-1/DNA complex composition whereas the IL-6 promoter activity was enhanced by TNF-α irrespective of the presence of an AP-1 binding site. The results demonstrate that the basic and activated IL-6 promoter activity is differentially regulated in the various p53 clones possibly due to alterations in the AP-1 composition. It had been noticed before that in fibroblasts, wt-p53 inhibited, whereas the p53val135 mutant form stimulated the expression of the c-fos and c-jun promoters. It has never been reported, however, that the expression of other Fos and Jun family proteins or their participation in the AP-1/DNA consensus of the IL-6 promoter is under influence of p53.

Loss of negative growth control by growth factors is another aspect of tumor development. TNF-α can be regarded as the most intensively studied negative growth control factor. The results presented in this study demonstrate a differential regulation of the IL-6 promoter and thus a differential loss of negative growth control by TNF-α.
regulator of solid tumors. Resistance, or loss of sensitivity, to TNF-α, can be associated with several mechanisms. One of the factors involved in resistance of tumor cells to TNF-α is the expression of c-erbB2. In chapter 5, the relationship between TNF-α sensitivity and doxorubicin-resistance of tumor cells was studied in a doxorubicin-resistant cell line panel consisting of the parental human small cell lung carcinoma cell line GLC₄ plus GLC₄-Adr₂ₓ and GLC₄-Adr₃₅₀ₓ with respectively doxorubicin resistance factors of 2 and 350 compared to GLC₄. Previously, the cell lines used in this study have been extensively examined and several mechanisms leading to doxorubicin-resistance have been identified. Compared to the parental cell line GLC₄, the highly doxorubicin-resistant GLC₄-Adr₃₅₀ₓ which is P-glycoprotein (P-gp) negative has been shown to exhibit an increased DNA-repair, increased detoxifying capacity, overexpression of the multidrug resistant associated protein (MRP) and of the lung resistance protein (LRP). In addition, GLC₄-Adr₃₅₀ₓ demonstrated decreased topoisomerase-II (Topo-II) activity due to decreased Topo-IIα and IIß expression. At the highest dose of 1000 ng/ml TNF-α, GLC₄ was almost completely resistant to TNF-α, whereas growth inhibition was observed in GLC₄-Adr₂ₓ (37%) and GLC₄-Adr₃₅₀ₓ (68%). Sensitivity to TNF-α appeared to correlate inversely with the expression and gene copies of Topo-IIα in these cell lines. The gene encoding for c-erbB2 is in the proximity of the Topo-IIα gene and its product is known to be one of the mechanisms of resistance to TNF-α. The doxorubicin-resistant cell lines with decreased Topo-IIα gene copies have a simultaneous decrease in c-erbB2 gene copies, probably due to linkage between these two genes. The reduced number of c-erbB2 gene copies coincided with decreased c-erbB2 expression and subsequently in increased sensitivity to TNF-α.

Additionally, it was analyzed whether other mechanisms were associated with TNF-resistance in GLC₄. There was no difference in TNF receptor-1 expression between the cell lines. Compared to the TNF-sensitive GLC₄-Adr₃₅₀ₓ, GLC₄ appeared to have a decreased activation of NF-κB after exposure to TNF-α which might indicate a reduced TNF-receptor function. In GLC₄ increased Bcl-2 expression was found, a protein described to confer TNF-α resistance. Moreover, it was demonstrated that combining TNF-α with the apoptosis associated poly ADP-ribose polymerase inhibitors 6-aminonicotinamide and 3-aminobenzamide did not affect TNF-α sensitivity in GLC₄ and GLC₄-Adr₃₅₀ₓ, excluding a pivotal role of poly ADP-ribose polymerase in TNF-α resistance in these cell lines.

These data show that doxorubicin-resistant tumor cell lines with decreased Topo-IIα gene copies can become sensitive to TNF-α probably due to loss of c-erbB2 gene copies. Furthermore, we found that several mechanisms associated with c-erbB2 overexpressing contribute to TNF-α resistance in GLC₄.

Multidrug resistance is the phenomenon that tumor cells are resistant to a group of natural drugs such as, anthracyclines, vinca-alkaloids, and epidophyllotoxins. Several mechanisms involved in multidrug resistance have been identified of which overexpression of P-glycoprotein, and multidrug resistance-related protein-1 account for increased drug efflux. Another mechanism of multidrug resistance is a decrease in the drug target Topo-II. In an attempt to overcome multidrug resistance, several modulators have been explored, among which the P-gp blockers, verapamil, cyclosporin A, and PSC 833. Several growth factors have shown to be potent proliferation stimulators of malignant hematopoietic cells. Furthermore, colony stimulating factors were shown to stimulate proliferation and to upregulate the expression of Topo-II and sensitize malignant blasts to chemotherapeutic drugs. In chapter 6, a growth factor-dependent erythroid leukemic cell line TF-1 was treated with IL-3 in order to attempt to modulate its sensitivity to etoposide, a chemotherapeutic agent frequently used in the treatment of patients with AML. The results demonstrated that an augmented cell death occurred...
when TF-1 cells were pre-incubated for 24 hours with IL-3 followed by 1 hour of treatment with etoposide. The increased cell death could not be ascribed to an increased number of apoptotic cells. The IL-3 treatment coincided with an upregulation of DNA Topo-IIα at mRNA and protein level after 24 hours, which was preceded by an upregulation of the oncogenic c-myc mRNA. In contrast, Topo-IIβ did not demonstrate an upregulation at mRNA level in response to IL-3. In addition it was shown that the increase in Topo-IIα resulted in a higher number of cleavable DNA complexes in cells treated with IL-3 followed by etoposide. This was not due to an increased etoposide uptake, since intracellular etoposide concentrations were not affected by IL-3. These data indicate that IL-3 modulates the etoposide cytotoxicity by upregulation of the etoposide target Topo-IIα in TF-1 cells.

In chapter 7, the concept of growth factor-modulated drug cytotoxicity was further explored. The study combines the administration of IL-3 and the P-gp blocker PSC 883 in order to modulate the cytotoxic effects of mitoxantrone and daunorubicin on two P-gp positive myeloid leukemic cell lines Mo-7 and GF-D8. Increased cytotoxicity occurred in Mo-7 cells pre-incubated for 24 hours with IL-3 followed by mitoxantrone or daunorubicin. Similar results were obtained for the GF-D8 cell line. The IL-3 administration did not affect P-gp and the apoptosis-inhibitor bcl-2 protein expression, cellular mitoxantrone concentration or mitoxantrone efflux. It did, however, coincide with an increase in the % of cells in S phase and Topo-IIα mRNA and Topo-II activity especially in the Mo-7 cell line. PSC 833 enhanced daunorubicin cytotoxicity in both cell lines. The combined administration of IL-3 and PSC 833 in the Mo-7 cell line resulted in an additive effect on daunorubicin cytotoxicity. The additive effect of IL-3 and PSC 833 was most pronounced in GF-D8 cells, which had also the highest P-gp expression. In contrast PSC 833 did not modulate the mitoxantrone effects, irrespective of the presence of IL-3. In summary the results demonstrate that the combined administration of IL-3 and PSC 833 did enhance the cytotoxic effects of daunorubicin but not mitoxantrone in two P-gp positive cell lines whereas the effects of mitoxantrone could be modulated by factors that influence Topo-II activity.

Perspectives
In this thesis three growth factor associated phenomena, expression of the autocrine growth factor IL-6 in human ovarian carcinoma, resistance to the growth suppressing activity of TNF-α, and growth factor-modulated drug resistance in acute myeloid leukemia are described.

Human ovarian tumors that express TNF-α show a better response to cisplatin-containing chemotherapy. Understanding the mechanisms underlying IL-6 expression in human ovarian tumor cells, might in the future, hand points for intervention, for example, by decreasing autocrine IL-6 secretion. Since NF-κB was found to be an important factor in the regulation of IL-6 expression in human ovarian cancer cell lines, down-regulation of the nuclear NF-κB expression with growth factors or antisense p-65 might be of potential interest. In addition it is potentially useful to restore the wt-p53 phenotype by transfection of wt-p53 or by m-p53 anti-sense therapy. In this way, not only autocrine pathways could be affected but the programmed cell death pathways could also be restored which might increase the sensitivity of tumor cells to chemotherapy. Currently, clinical studies with p53 gene therapy, also in ovarian carcinoma, are ongoing. Suppression of endogenous IL-6 expression by, for instance, antisense oligonucleotides might be feasible, but has not yet been assessed. In cell line and animal models, antisense strategies, directed to other growth factor and their receptors are explored. Examples are antisense strategies directed at insulin-like growth factor I and II and its receptor in ovarian cancer cells (3, 4), vascular endothelial growth factor in melanoma...
cells (5), HER2/neu in breast carcinoma cells (6), and epidermal growth factor receptor in head and neck squamous cell carcinoma (7), renal cell cancer (8), and colon carcinoma (9, 10).

We showed that various p53 transfectants in the human ovarian A2780 cell line display AP-1/DNA complexes with different Fos and Jun family proteins. It is still unclear how p53 precisely affects the AP-1 composition. Further analysis might reveal whether these differences are due to altered gene expression or phosphorylation of the subunits.

Combination therapy of TNF-α with melphalan in isolated limb perfusion demonstrated distinct anti-tumor effects (11, 12). To hit various targets instead of just one is explored more and more in anti-cancer treatment. This idea was also tested in vitro, in order to circumvent multidrug resistance with growth factors and P-gp inhibitors.

The application of IL-3 and other growth factors have been shown useful to increase sensitivity of acute myeloid leukemic cell lines to various chemotherapeutic drugs in vitro. We and others have observed that the increased response induced by growth factors was associated with an increased number of cells in S phase and higher Topo-II mRNA and protein levels. Additional studies should reveal whether this approach is useful in the clinic and whether a combination of growth factors added to MDR-related chemotherapeutic drugs would be more effective in the circumvention of multidrug resistance. Co-administration of IL-1 and IL-3 could induce proliferation of the heterogeneous population of early and more committed acute myeloid leukemia blasts in vivo. Furthermore, besides TNF-α and the hematopoietic growth factors, IL-6 and IL-1 might also be applied in vivo to sensitize solid tumor cells, such as ovarian tumor cells, to Topo-II directed drugs. Intensive studies could reveal additional mechanisms and keys to improve the use of growth factors in anti-cancer therapies.

References