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Clinical and laboratory studies on chemo-resistance and -sensitivity, and the role of TNF

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Chapter 11

SUMMARY AND CONCLUSIONS

In this thesis several aspects of anti-cancer therapy have been described. Despite the application of many forms of chemo- or immunotherapy in many tumor types, most patients are not curable yet. However, since the introduction of platinum-containing multidrug regimens for the treatment of patients with disseminated germ-cell cancer, this type of cancer has become a model for a curable neoplasm. In the **Introduction**, the current treatment of disseminated germ-cell cancer of the testis is described and several research questions regarding the efficacy and toxicity of chemotherapeutic regimens for this kind of cancer are formulated. Some of the side-effects of bleomycin, a widely applied drug in the treatment of disseminated germ-cell cancer, seem to be related to induction of tumor necrosis factor-α (TNF). Because TNF can also act as an anti-tumor agent, research questions are formulated addressing the feasibility of TNF as an anti-tumor agent, particularly in drug-resistant tumor cell lines.

Chapter 1 describes the efficacy and toxicity of a combination regimen consisting of cyclophosphamide, vincristine, and carboplatin, a cisplatin analogue, (COC) for the treatment of advanced seminoma on an outpatient basis. Twenty-seven patients of which 6 had been treated previously with radiotherapy, received four cycles with chemotherapy at 3-week intervals. A complete response (CR) was observed in 6 patients (95% confidence interval (CI), 6-38%), a partial response (PR) in 19 patients (95% CI, 51-88%) and in 2 patients (95% CI, 0-18%) only a decline in tumor markers was seen whereas no reduction in retroperitoneal mass occurred. Post-chemotherapeutic masses were not surgically removed or irradiated. Two patients have died after a median follow-up of 26 months (range 5-69 months) yielding 93% of the patients with no evidence of disease, a figure equivalent to that achieved by other polychemotherapeutic regimens in this patient population. Main toxicity was haematological with 100% of the patients experiencing leucocytopenia grade III/IV and 81% thrombocytopenia grade III/IV. Non-haematological side-effects were rare. In conclusion, COC is an effective regimen for the treatment of advanced seminoma with the advantage that it can be applied on an outpatient basis. In the future, its main toxicity, myelosuppression, may be partly prevented by the addition of growth factors.

However, despite the successful application of chemotherapy in the treatment of disseminated germ-cell cancer, there are still patients who either do not achieve a

condition of no evidence of disease or relapse after an initial response. By salvage chemotherapy, even some of these patients can be cured. In **chapter 2**, a small study is presented on the efficacy and toxicity of such a salvage treatment. Eight patients with relapsed non-seminomatous testicular cancer after primary cisplatin-containing chemotherapy were treated with high dose methotrexate, vincristine, and cisplatin. Two patients obtained a complete response (CR) (43^+ and 53^+ months), 2 a partial response (PR), whereas no alterations in tumor mass or tumor markers were observed in the other 4 patients. Toxicity was mainly due to methotrexate and could be ameliorated to a large extent by leucovorin. In conclusion, this salvage therapy yields a response rate equivalent with that achieved by other salvage regimens and has considerable anti-tumor activity in patients with relapsed non-seminomatous germ-cell cancer with manageable side-effects.

The main drawback of the application of chemotherapy is the occurrence of side-effects. In the treatment of disseminated germ-cell cancer, bleomycin is a cytotoxic drug used in many regimens and is feared for its induction of bleomycin-induced pneumonitis (BIP) which is sometimes fatal. Therefore, it is important to detect pulmonary alterations due to bleomycin early. Because bleomycin is nowadays only applied as part of a multidrug regimen and not as a single agent, it is not possible to establish pulmonary function tests which are specific for bleomycin-induced pulmonary changes. The study described in **chapter 3** was performed to establish the changes in pulmonary function in good risk patients with disseminated non-seminomatous germ-cell cancer randomised to receive treatment with four cycles of either etoposide, and cisplatin (EP) (27 patients) or etoposide and cisplatin with the addition of 30 mg bleomycin i.v. weekly for 12 weeks (BEP) (27 patients). This enabled us to determine whether lung function assessments are specifically affected by bleomycin. Lung functions determined before and at 3-week intervals during chemotherapy, consisted of the transfer capacity of the lungs for carbon monoxide (T_{LCO}), the diffusing capacity of the alveolo-capillary membrane (D_M), the pulmonary capillary blood volume (V_C), the transfer capacity of the lungs for carbon monoxide per unit alveolar volume (K_{CO}), and the slow inspiratory vital capacity (VC). During treatment, both groups showed a decline in T_{LCO}, D_M, and K_{CO}, but differences in these parameters between the groups were not recorded. However, the VC and V_C decreased significantly in the patients during treatment with BEP, but remained constant in the EP group. Therefore, it could be concluded that the T_{LCO}, D_M, and K_{CO} are not proper tools to detect pulmonary damage by bleomycin as part of this multidrug chemotherapeutic regimen. VC and V_C however, are pulmonary function tests which reflect specifically pulmonary alterations due to bleomycin.

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cisplatin is nephrotoxic leading to a change in pharmacokinetics of bleomycin, the combination of these two agents not only yields a more effective anti-tumor activity but may also enhance the bleomycin-induced pulmonary toxicity. In **chapter 4**, we explored whether a decrease in renal function due to cisplatin augmented bleomycin-induced pulmonary function in a group of patients with non-seminomatous testicular cancer receiving etoposide, and cisplatin with (BEP) or without bleomycin (EP). Before and at 3-week intervals during treatment, creatinine clearance and pulmonary functions were determined. Compared to pretreatment, both groups showed a similar decline in renal function which was attributed to the use of cisplatin. In the BEP group, the decrease in renal function was significantly correlated with a decrease in the transfer capacity of the lungs for carbon monoxide (T$_{LCO}$) and the vital capacity (VC), parameters known to reflect bleomycin-induced pulmonary toxicity. Other pulmonary functions studied did not correlate with renal function. In the 27 patients receiving etoposide and cisplatin (EP) being the control group, no relationships between changes in renal function and pulmonary function assessments were observed at all. These data suggest that there is indeed an augmented toxic effect of bleomycin on the lungs when renal function is impaired. Therefore, special attention for the development of bleomycin-induced pulmonary toxicity should be paid when bleomycin is combined with a nephrotoxic agent such as cisplatin.

Besides pulmonary toxicity, the application of bleomycin is also featured by the occurrence of acute side-effects such as fever, chills, and hypotension observed directly after administration of this agent. There are several indications that induction of cytokines by bleomycin is involved in the development of these acute side-effects as well as in the induction of pulmonary damage. In **chapter 5**, the effect of bleomycin infusion on circulating levels of the cytokines tumor necrosis factor-α (TNF), interleukin-1ß (IL-1ß), and transforming growth factor-ß (TGFß) is studied in 14 patients receiving 30 mg bleomycin infused in 15 minutes. Compared to pretreatment, the mean TNF plasma level appeared to be increased significantly 3, 4.5, and 24 hours after administration. No effects of bleomcyin were observed on levels of IL-1ß and TGFß. These data suggest that induction of cytokines, and TNF in particular, is indeed involved in the pathogenesis of bleomycin-induced side-effects.

Besides being a mediator of side-effects, TNF has also the ability to act as an anti-cancer agent. As an single agent TNF possesses anti-tumor activity against a wide range of tumor cell types whereas non-transformed cells are relatively resistant. The observation that in animals TNF is also active as an anti-tumor agent, raised high expectations for its clinical application. However, phase I and II trials with TNF as single agent were featured by severe side-effects and almost no anti-tumor activity. Recently, the interest in TNF was revived. In the setting of hyperthermic

isolated limb perfusion (HILP), TNF combined with the alkylating agent melphalan resulted in dramatic anti-tumor effects against melanomas and sarcomas, tumors known to be highly resistant to cytotoxic agents. A review on TNF as anticancer agent is presented in **chapter 6**.

In vitro, the relationship between resistance of tumor cell lines to cytotoxic agents and sensitivity to TNF has been the subject of many studies. For tumor cell lines resistant to doxorubicin, a widely used anti-cancer drug, both resistance and an increased sensitivity to TNF has been described. The mechanism responsible for the latter phenomenon is unclear. In **chapter 7**, we explored the relationship between TNF-sensitivity and doxorubicin-resistance in our widely examined doxorubicin-resistant cell line panel consisting of the parental cell line GLC_4 and its sublines GLC_4-Adr_{2x} and GLC_4-Adr_{350x} with respective resistance factors of 2- and 350-fold. One of the mechanisms responsible for the doxorubicin-resistance in GLC_4-Adr_{2x} and GLC_4-Adr_{350x} is a decreased topoisomerase IIα gene copy number resulting in a decreased topoisomerase IIα protein level, the target of doxorubicin. In our study, we found that GLC_4 was almost complete resistant to TNF, whereas a maximum growth inhibition of 37% and 68% was observed in GLC_4-Adr_{2x} and GLC_4-Adr_{350x} respectively. In these cell lines, sensitivity to TNF appeared to correlate inversely with topoisomerase IIα expression and gene copies. In the proximity of the topoisomerase IIα gene on chromosome 17q is located the gene encoding for c-erbB2 which product is a known cause for TNF-resistance. We demonstrate that GLC_4-Adr_{2x} and GLC_4-Adr_{350x} which have compared to GLC_4 a decreased topoisomerase IIα gene copy number, have a simultaneous lower number of c-erbB2 gene copies (75% and 65% of GLC_4 respectively), probably due to physical linkage between these two genes. During exposure of GLC_4 to doxorubicin to establish GLC_4-Adr_{2x} and GLC_4-Adr_{350x}, this linkage resulted not only in cell lines with decreased topoisomerase IIα gene copy numbers but also with reduced c-erbB2 gene copies. This reduced number of c-erbB2 gene copies in GLC_4-Adr_{2x} and GLC_4-Adr_{350x} leads to a decreased c-erbB2 expression (55% and 35% of GLC_4) and subsequently in increased sensitivity to TNF. Furthermore, we established some of the mechanisms associated with c-erbB2 overexpression resulting in TNF-resistance in GLC_4. We found that compared with the TNF-sensitive GLC_4-Adr_{350x}, GLC_4 had a 5-fold increased bcl-2 expression and probably a decreased TNF-receptor functionality, both factors known to confer resistance to TNF. These in vitro experiments suggest enhanced efficacy of TNF in tumor cell lines resistant to doxorubicin yielding a possible tool to circumvent drug-resistance.

In vitro, TNF exerts its cytotoxicity at least partly by induction of apoptosis. One of the most important genes mediating apoptosis is the tumor suppressor gene p53. Wild-type p53 (wtp53) is induced after DNA-damage resulting in either

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apoptosis or G1 and G2/M-cell cycle arrest facilitating DNA-repair. In about 50-60% of the human tumors, the gene encoding for p53 is mutated. This mutated p53 (mtp53) may affect the normal function of p53 and subsequently alter the sensitivity of these tumors to cytotoxic agents. In **chapter 8** we establish the effect of p53 status on sensitivity to TNF in a model consisting of a human ovarian carcinoma cell line containing wtp53 transfected with a control plasmid (A2780/cmv) or with a plasmid constitutively expressing a commonly occurring form of mtp53 (A2780/m248). We found that A2780/cmv was relatively insensitive to TNF (maximum growth inhibition 30%), whereas A2780/m248 showed increased susceptibility for TNF (maximum growth inhibition 67%). Exploring the mechanisms responsible for the enhanced sensitivity of a mtp53 containing tumor cell line revealed that compared to A2780/cmv, A2780/m248 had a reduced expression of p53-dependent genes such as bcl-2 and c-erbB2 (42% and 54% of A2780/cmv respectively), both factors known to confer TNF-resistance. Therefore, it is concluded that p53 status can indeed affect sensitivity to TNF and that reduced expression of p53-dependent genes resulting in resistance to TNF, may be responsible for the enhanced sensitivity to TNF in the mtp53-containing cell line.

Clinical as well as preclinical evidence suggests that TNF is able to overcome drug resistance in tumors. In order to elucidate whether TNF is able to do so in tumor cell lines which are resistant due to a mutation in the tumor suppressor gene p53, we established in **chapter 9** the in vitro combined cytotoxic effects of TNF with various cytotoxic agents in a model consisting of a human ovarian cancer cell line containing wtp53 and sublines which were made resistant to various agents by transfection of a mutated p53. We found that compared to the control cell line A2780/cmv, two sublines containing mtp53 showed increased resistance against various cytotoxic agents but also collateral sensitivity to TNF. The interaction of TNF with the cytotoxic agents tested was similar in drug-sensitive as well as drug-resistant cell lines. However, due to increased sensitivity in A2780/m248 to TNF at the dose used for the combinations, the combination of TNF with several cytotoxic drugs reduced the level of resistance against these cytotoxic agents in A2780/m248 compared to the control cell line A2780/cmv. So, in conclusion, this study shows that addition of TNF can ameliorate resistance to cytotoxic agents in tumor cell lines resistant due to the presence of mtp53. This reduction in resistance by TNF is not due to synergistic interaction, but to collateral sensitivity to TNF in drug-resistant cel lines, a phenomenon which has been described in several other reports.

Besides preclinical studies as described in chapter 7-9, also clinical studies exploring TNF as an anti-tumor agent in the setting of hyperthermic limb perfusion (HILP) showed that TNF is active against chemotherapy-resistant tumors. Furthermore, these studies of HILP with TNF and melphalan revealed that by

applying intensive care technology, higher systemic TNF levels can be reached than previously reported in phase I trials administrating TNF systemically. Based on these observations, it might be feasible by applying such technology to administer systemically TNF in high doses in combination with cytotoxic drugs to patients with disseminated cancer. However, it is important before initiating such studies to establish the effects of such high doses of TNF in combination with cytotoxic drugs on vital organs such as the lungs. In **chapter 10**, the impact on the lungs of such a treatment was studied by performing pulmonary function assessments in 12 patients before and after HILP with TNF and melphalan. Due to leakage during perfusion, systemic TNF levels were reached up to 356 ng/ml. Significant alterations in the vital capacity (VC), the capillary blood volume (Vc), the diffusing capacity of the alveolo-capillary membrane (Dm), and the transfer capacity of the lungs for carbon monoxide per unit alveolar volume (K_{CO}) were observed 1 week after HILP. Eight weeks after perfusion all disturbances had returned to pretreatment level. Alterations in pulmonary function one week after perfusion were not related to maximum TNF-levels. In conclusion, this study shows that high systemic TNF-levels in combination with melphalan causes transient pulmonary function disturbances. These disturbances which are probably partly caused by the high systemic TNF levels, return to pretreatment level eight weeks after treatment. Therefore, these results would not preclude further studies on the feasibility of TNF as a systemic agent with a starting dose level to be calculated from the leakage serum levels.

We can therefore conclude that TNF, being a mediator of some of the side-effects of bleomycin, also can be used to circumvent drug-resistance in tumor cells. Furthermore, toxicity of high doses of systemically administered TNF can be managed adequately in the future by applying intensive care technology.

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