A potential supportive role of macrophages in the treatment of ovarian cancer.
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Document Version
Publisher's PDF, also known as Version of record

Publication date:
1999

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

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CHAPTER 8

Summary

The aim of the studies presented in this thesis was to explore and to exploit the tumor cytotoxic potential of macrophages in immunotherapeutic treatment of ovarian cancer. For this purpose, we performed in vivo studies with non-tumor-bearing mice (chapters 3 and 4) as well as with mice bearing a murine ovarian tumor (chapters 4 and 5). In addition, in vitro studies with human ovarian tumor cell lines and human ovarian tumors were performed (chapters 6 and 7).

Chapter 2 encompasses a literature review concerning the involvement of macrophages in tumor biology in general. Macrophages, when activated by bacterial cell wall components or certain cytokines, are able to specifically recognize and kill tumor cells. Among the mediators capable of direct cell killing are tumor-necrosis factor (TNF) and nitric oxide (NO). Macrophages populate areas both within and around tumors, often in large numbers, indicating that these cells are recruited to sites of tumor growth. Tumor cells can secrete mediators that on the one hand attract macrophages and on the other are able to suppress their activity. These seemingly opposite observations illustrate our limited knowledge of the role and function of tumor-associated macrophages. The state of activation of the macrophages (e.g. when macrophage are in a primed condition) probably determines to a large extent whether macrophages are allowed to lyse tumor cells by secreting cytotoxic mediators or rather stimulate tumor growth by secreting mediators such as IL-10 which are involved in angiogenesis. This process of blood vessel formation is necessary for tumors to progress beyond a certain initial stage. The ambiguous role of macrophages in tumor development calls for an immunotherapeutic treatment aimed at macrophage activation. A number of clinical phase I and II trials based on activated macrophages or subpopulations of macrophages (i.e. dendritic cells) have already been performed, others are still ongoing. It should be noted, that although this kind of immunotherapy is expected to be beneficial for patients with minimal residual disease, only patients with advanced disease are enrolled in phase I and II clinical studies. At that late stage of the disease, inaccessibility of the tumor by macrophages, low macrophage to tumor cell ratio’s and immunosuppression of the patients are major obstacles for immunotherapy to be effective. This presumably explains so far, although immune responses have been described, no complete remission have been obtained in these clinical studies.

Chapter 3 describes the effects concerning the cytotoxicity of peritoneal cells upon intraperitoneal (i.p.) injections of recombinant murine granulocyte-macrophage colony-stimulating factor (rmGM-CSF) in non-tumor bearing mice. Upon i.p. injections with rmGM-CSF the number of peritoneal cells increased. The increase in cell number was mainly accounted for by an increase in the number of macrophages. The peritoneal cells were highly cytotoxic upon additional in vitro stimulation with lipopolysaccharide (LPS). This was in contrast with peritoneal cells isolated from control mice treated with phosphate-buffered saline, that were only moderately cytotoxic upon LPS stimulation in vitro. The cytotoxic capacity was strongly correlated with the amount of NO secreted by these cells. As the peritoneal cells consisted for 80% of macrophages, the results obtained can presumably be attributed to this cell population. The results of this study indicate that i.p. injections with rmGM-CSF can lead to recruitment of a new monocyte or macrophage population in the peritoneal cavity.
SUMMARY AND FUTURE PERSPECTIVES

In chapter 4 we describe the cytotoxic properties of peritoneal cells upon i.p. injections of liposomal muramyltripeptide phosphatidylethanolamine (L-MTP-PE) in non-tumor bearing mice. Injections with L-MTP-PE caused a strong increase in the number of peritoneal cells, mainly due to an increase in the number of peritoneal macrophages and to a minor extent due to an increase in the number of neutrophils. These peritoneal cells were highly cytotoxic to tumor cells after in vitro stimulation with LPS, in contrast to control peritoneal cells. Because placebo liposomes also induced a moderate increase in the number of peritoneal cells and cytotoxic potential of these cells, we conclude that certain lipid components in the liposomes, such as phosphatidylserine, may by themselves influence the immune system. Secondly, we investigated the effects of combined i.p. treatment with L-MTP-PE, GM-CSF and cisplatin using different treatment schedules in mice bearing a murine ovarian tumor. Despite the large numbers of peritoneal macrophages present in the peritoneal cavity, no prolonged survival was observed with any of the treatment schedules tested. Remarkably, however, was the inhibitory effect of a large injection volume on tumor growth. Multiple daily injections with 0.5 ml of diluent within a few days after tumor inoculation, strongly inhibited tumor growth. Taking these results together, we believe that the tumor cells used in this murine ovarian tumor model are too rapidly-growing to allow the observation of an antitumor effect resulting in a prolonged survival in these mice.

In chapter 5 we describe a different approach to achieve an i.p. immunotherapeutic macrophage-mediated immunotherapeutic treatment. We injected the rmGM-CSF gene, cloned into an expression vector based on the Semliki Forest virus (SFV) replicon, i.p. into mice. Thus, we expected to achieve prolonged secretion of the recombinant protein over a period of several days. Initial results with i.p. injections of SFV particles encoding for the luciferase reporter gene, indicated that transfection of cells after i.p. injections with SFV particles predominantly occur in the tumor cells in the peritoneal cavity of tumor-bearing animals and in the peritoneal lining. This would mean that most rmGM-CSF produced by transfected cells can be expected to be released into the peritoneal cavity. In contrast to i.p. injections with soluble rmGM-CSF, we observed that upon in vivo transfection with SFV-GM-CSF particles the peritoneal cells were tumor cytotoxic without additional in vitro stimulation with LPS. When tumor-bearing mice were treated with SFV-GM-CSF particles i.p., inhibition of tumor growth was observed during the first 12 days after tumor inoculation. Nonetheless, the overall survival of the mice was not significantly prolonged upon in vivo transfection with the rmGM-CSF gene. Taken together, our results indicate that i.p. injections with SFV-GM-CSF particles leads to rmGM-CSF production by transfected cells in the peritoneal cavity. Transfection with virus particles, can induce death of the transfected cells. This may, conceivably, give rise to the production of cell debris which in turn may elicit an immunological response. Thus, the presence of virus particles may indirectly be responsible for the induction of an immune reaction which, together with rmGM-CSF secretion by transfected cells, might be responsible for the in vivo activation of peritoneal macrophages.

While the previous chapters describe the cytotoxic potential of murine peritoneal macrophages, chapters 6 and 7 mainly focus on the potential role of human monocytes and macrophages in relation to human ovarian tumor cells and human
ovarian cancer. In chapter 6 we investigated the potential of human monocytes to lyse human ovarian tumor cell lines. These cell lines following transfection with plasmids containing mutated p53 genes, expressed several mutations in the p53 tumor suppressor gene, which is involved in apoptosis. Due to the expression of various mutant p53 proteins these cell lines differed in their sensitivity to cytostatic drugs and TNF. We showed that monocytes isolated from healthy volunteers were able to efficiently lyse the tumor cells. Cell killing could partly be attributed to TNF and NO secretion. Also peritoneal macrophages isolated from rmGM-CSF and L-MTP-PE treated mice were able to lyse tumor cells. Macrophages isolated from L-MTP-PE treated mice were cytotoxic to tumor cells without additional in vitro activation, in contrast to macrophages isolated from rmGM-CSF treated mice. Apparently, L-MTP-PE is able to activate peritoneal macrophages directly to tumor cytotoxicity. These results indicate that the mechanism of tumor cell lysis by activated monocytes proceeds independent of the p53 apoptotic route. This leads us to conclude that immunotherapeutic treatment based on monocyte/macrophage activation may provide a cotreatment modality of ovarian tumors that have become resistant to conventional chemotherapeutics.

Finally, in chapter 7 we determined in an immunohistochemical study the expression of two inducible enzymes in surgically obtained material of human ovarian tumors. Both enzymes have been described to be expressed in some tumors as well as in activated macrophages. The inducible enzyme cyclo-oxygenase 2 (COX-2) is responsible for the production of the macrophage suppressive mediator prostaglandin E₂ (PGE₂), while the inducible type of NO-synthase (iNOS) is responsible for the production of NO. NO is one of the mediators directly involved in cell killing but it can also function as a vasodilator. The latter function may therefore provide a favorable condition in tumor development. We investigated the expression of these enzymes in epithelial stroma of malignant, borderline, and benign ovarian tumors and in tumor-associated macrophages. Expression of COX-2 was observed in the epithelial stroma of a majority of the malignant, borderline as well as benign tumors, while in a minority of the tumors, iNOS expression in the epithelial stroma cells was found. Most tumors also contained macrophages. There was a correlation between the grade of malignancy of the tumor and the number of macrophages found in and around the tumor; the largest numbers of macrophages were encountered in the malignant tumors. Macrophages positive for COX-2 or iNOS were only observed in some malignant and borderline tumors, but not in benign tumors. The COX-2 and iNOS positive macrophages represented only small subpopulations of the total tumor-associated macrophage population. Taken together, our results show that COX-2 expression is not limited to malignant tumors, but can also be expressed in epithelial cells of non-malignant tumors. Furthermore, only a small population of the tumor-associated macrophages in some malignant and borderline tumors were positive for COX-2 or iNOS, indicating that most tumor-associated macrophages were not in an activated state.

In conclusion, exploring the potential of the peritoneal macrophage population to lyse peritoneal tumor cells we found that, although the peritoneal macrophage population could be considerably expanded, and stimulated to tumor cytotoxicity, this did not result in a prolongation of survival in the murine ovarian tumor model used in this study. Nonetheless, the activation of peritoneal macrophages upon i.p.
administered the GM-CSF gene in a viral vector and the observation that macrophages and monocytes are able to lyse tumor cells irrespective of their p53 status provides useful information for the further development of new immunotherapeutic treatments of ovarian cancer.

**Future perspectives**

The results presented in this study show that upon i.p. treatment with either GM-CSF and L-MTP-PE the number of peritoneal macrophages in mice increased significantly. In addition, the tumor cytotoxic capacity of these macrophages was increased compared to that of control animals. Although peritoneal macrophages derived from L-MTP-PE-treated animals were directly cytotoxic towards some tumor cell types, peritoneal macrophages isolated from GM-CSF-treated mice always needed an additional stimulation to become fully tumor cytotoxic. Therefore, an interesting finding described in this thesis is the direct activation to tumor cytotoxicity of peritoneal macrophages upon GM-CSF producing virus particles. This effect was not observed upon injections with soluble GM-CSF, non-encoding virus particles or virus particles encoding an irrelevant protein. We therefore hypothesize that in our experiments the viral vector, used in the in vivo transfection with the GM-CSF gene, by itself serves as a trigger for macrophage activation, additional to the GM-CSF produced by the transfected cells. These results may have implications for gene therapeutic strategies in cancer therapy, considering the ongoing debate on whether non-viral or viral vectors should be used in gene therapeutic treatment. The observation that a viral vector by itself can induce an additional immune response, indicates that the use of a viral vector may provide a favorable condition in gene therapy that aims at the activation of the immune system.

Furthermore, *in vitro* experiments showed that cells of human ovarian tumor cell lines resistant to chemotherapeutics, based on p53 mutations were efficiently killed by human monocytes. These monocytes could be further activated to tumor cytotoxicity with GM-CSF and/or LPS. Remarkably, as NO and TNF are considered as the most important mediators in tumor cell killing, inhibition of NO-synthesis in monocytes and neutralization of TNF secreted by monocytes with anti-TNF antibodies only resulted in a minor decrease in monocyte-mediated cell killing. These results indicate, that also other pathways are operational in tumor cell lysis by (human) monocytes. Over the past decade, new members of the TNF and TNF-receptor family have been discovered among which are FasL/Fas and TRAIL/TRAIL-receptor. Both pathways function via a ligand/receptor binding. Until now, their role in monocyte/macrophage-mediated tumor cytotoxicity has not yet been investigated. Therefore it would be interesting to elucidate the potential role of these or other unknown mediators in cell killing by monocytes or macrophages.

Overall, our results show that macrophages or monocytes can have a supportive role in immunotherapeutic treatment of ovarian cancer. A major problem to overcome might be the recruitment of a sufficient number of macrophages to kill large numbers of tumor cells. Adoptive cellular therapy, in which monocytes can be isolated from the peripheral blood with large numbers, and are administered to the patients after activation *in vitro*, might provide a solution. It will, however, require extensive
additional studies to achieve an immunotherapeutic treatment based on macrophage or monocyte activation that significantly adds to currently obtained chemotherapeutic results.