Summary

Although, melanoma accounts for only 4% of all skin cancers it is the major cause of skin cancer related deaths. Clinical outcome in melanoma patients depends on several variables of which tumour thickness is one of the most important.[1] Other important prognostic factors include ulceration, location of primary tumour, lymphatic invasion, gender and age.[2,3,4] Melanomas are thought to be immunogenic tumours partly because approximately 2 to 4% of all patients with a primary melanoma[5,6,7] and about 9% of patients with melanoma with lymph node metastasis do not have an identifiable primary lesion. This is explained by the hypothesis that the primary tumour regressed spontaneously through the presence of an active immune response.

Many studies have shown the importance of a functional cellular immune response in preventing melanoma dissemination. The cell death inducing effect of immune therapy largely depend on the induction of apoptosis in the tumour cells and also requires recognition of antigen presented in the context of the appropriate MHC molecules. Therefore, disruption of the apoptosis pathway, loss of antigen presentation and presence of local or systemic immune suppressive factors may be important causes for resistance to immune therapy.

Detecting disruption of the apoptosis pathway in melanoma cells and detecting possible causes of failure of the immune system to clear tumour cells from the body may be key requisites to overcome immune therapy resistance and improve clinical outcome. Determining the role of cells of the cellular immune system present in the tumour and the presence of apoptotic regulators expressed in the melanoma cells may provide us with new insights on how to induce apoptosis in melanoma cells or make the tumour cell more susceptible to immune therapy.
In this thesis, we investigated the presence of a cellular immune response in relation to clinical outcome, inhibition of the apoptosis pathway and to MHC class I and II expression on tumour cells. The results strongly suggest that the cellular immune response is indeed very important in preventing tumour cells from disseminating to lymph nodes and distant sites.

In **chapter 2** we describe that favourable outcome in clinically stage II melanoma patients is associated with presence of activated Tumour Infiltrating T-lymphocytes (TILs) and preserved MHC class I expression. Using immune histochemical analysis we show that presence of GrB^+ TILs, $CD4^+$ TILs and a putative intact antigen presentation in the context of expression of MHC class I on tumour cells and MHC class II expression on tumour infiltrating antigen presenting cells predicts a highly favourable outcome in clinically stage II melanoma patients.

Sentinel lymph node (SLN) status is strongly related to clinical outcome in melanoma patients and we investigated whether presence of activated and/or regulatory TILs predicts SLN status in clinically stage I/II melanoma patients. In **chapter 3** we show, using immune histochemical analysis that absence of GrB^+ TILs in primary melanoma biopsies predicts presence of a (SLN) metastasis. In here, we conclude that absence of GrB^+ TILs in primary melanoma biopsies is strongly associated with presence of SLN metastasis.

In **chapter 4** we describe that a high percentage of activated CTLs in primary melanoma is associated with a favourable clinical outcome, possibly reflecting immunogenic properties of the primary tumour. This is supported by the significant correlation detected between intact MHC class I expression and a high percentage of activated CTLs. Furthermore, we found that expression of the Granzyme B inhibitor PI-9 in metastatic melanoma predicts poor clinical outcome in vaccinated patients with stage III/IV melanoma and we hypothesised that the presence of PI-9 might explain why outcome after metastatic melanoma is not related to presence of high percentages activated CTLs.

In **chapter 5** we investigated which antibody and which staining protocol can be used for optimal detection of c-FLIP expression in paraffin-embedded tissue sections. Only two of four tested antibodies proved to be specific enough for reliable analysis of c-FLIP expression. Using these two antibodies and with the optimized protocol we could demonstrate expression of c-FLIP in Diffuse Large B-Cell Lymphoma and Hodgkin Lymphoma and not in other lymphoma types.

However, as described in the **addendum of chapter 5** we failed to detect expression of c-FLIP on paraffin embedded tissue of melanoma cells, although c-FLIP expression was detected at mRNA level by RT-MLPA analysis in isolated melanoma cells (see **chapter 5**). Thus, expression of c-FLIP at protein level in melanoma is most likely below the detection limit of the tested antibodies, indicating that expression levels in melanoma is below expression levels detected in lymphomas.

In **chapter 6** we establish that downstream disruption of the intrinsic apoptosis pathway is a key event in a subset of melanomas. Expression profiles of apoptosis regulating genes in melanoma cells isolated from patients from the ASI trial[8] were determined by RT-MLPA and correlated with clinical outcome. Functional integrity of the intrinsic apoptosis pathway was determined by measuring caspase activity after induction of apoptosis. We observed that activation of the intrinsic apoptosis pathway is a key event in a subset of melanomas, with concomitant disruption of this pathway downstream from caspase 9 activation. However, disruption of this pathway does not implicate poor outcome of melanoma patients following ASI therapy, suggesting that an intact intrinsic apoptosis pathway is not a prerequisite for CTLs and/or NK cells to kill the melanoma cells.

General discussion

1. An adequate cellular immune response may prevent dissemination of melanoma cells

In this thesis we repeatedly showed that presence of GrB^+ TILs in the primary tumour is correlated with a favourable clinical outcome of melanoma patients, which is also reflected by the observed association between absence of GrB^+ TILs and SLN metastasis **(chapter 3)**. The data suggest that an intact cellular immune response is one of the major factors determining clinical outcome in melanoma patients.

Favourable outcome in melanoma patients is not only associated with presence of activated TILs and preserved MHC class I antigen expression. We also observed that presence of $CD4^+$ TILs and putative intact antigen presentation in the context of MHC class II in primary melanomas is significantly correlated with a highly favourable outcome in clinically stage II melanoma patients (**chapter 2)**. $CD4^+$ TILs contain the $CD4^+$ T-helper population; however, they also contain a population of suppressive $CD4^+$ TILs.

2. Different immune escape mechanisms may explain poor outcome in melanoma patients

Tumour cells can use different mechanisms to evade the cellular immune system and the cellular immune system itself can be interrupted at different stages. One of these mechanisms frequently described is down regulation of MHC class I molecules.[9,10,11,12] Loss of MHC class I expression was frequently observed in the melanoma biopsies of both primary and metastasized tumour cells in our studies **(chapter 2, 4 and 3)**. We observed that loss of MHC class I expression correlated with absence of GrB^+ TILs, suggesting that absence of GrB^+ TILs is caused by defective antigen presentation resulting in inadequate activation (as reflected by lack of GrB expression) of cytotoxic T lymphocytes (CTLs).

Another escape mechanism might be expression of FoxP3: It was recently suggested that suppressive $FoxP3^+$ T-lymphocytes are involved in induction of immune tolerance in SLN metastases.[13,14] Our data support this point of view **(chapter 3).** When melanomas containing GrB^+ TILs in the primary tumour, were subdivided in melanomas with and without $FoxP3^+$ TILs, we observed that SLN metastases occurred most often in either melanomas without GrB^+ TILs or in melanomas with GrB^+ TILs and $FoxP3^+$ TILs. Thus, even though the immune response is powerful enough to activate the infiltrating lymphocytes, the $FoxP3^+$ suppressive TILs might cause local immune suppression facilitating tumour cell persistence. This observation might also explain that, despite presence of GrB^+ TILs in the SLN metastases the activated immune system is apparently incapable of killing the metastasized tumour cells, probably because most of these SLN metastases also harboured $FoxP3^+$ TILs. The significant correlation between favourable outcome and presence of high percentages of GrB^+ TILs was not observed in metastasized melanoma. We observed that the lack of correlation might at least partly be explained by presence of suppressive $FoxP3^+$ cells in the metastasis or by expression of PI-9. We observed that metastatic melanoma cells can express PI-9, which was found to be associated with poor clinical outcome in tumour cell vaccinated patients with stage III/IV melanoma **(chapter 4)**. Expression of PI-9,

the only known human intracellular GrB inhibitor,[15] in target cells may inhibit immune surveillance by blocking NK and CTL-induced cytotoxicity through the perforin/granzyme pathway and through the Fas/FasL pathway.[16] Whether expression of PI-9 in the tumour cells is sufficient to block cytolysis induced by CTLs and NK cells remains controversial.[17,18] We also observed relatively high levels of PI-9 mRNA expression in some patients with a favourable clinical outcome after ASI therapy **(Chapter 6)**. In these cases CTL and/or NK cell induced cell death might be induced by members of the TNF family, such as membrane-bound Fas ligand (FasL) that interacts with the Fas receptor or soluble- and membrane-bound TNF-related apoptosis-inducing ligand (TRAIL) that induces cell death via the TNF receptor (TNF-R1) and TRAIL receptors (TRAIL-R1 and -R2).[19]

The cell death inducing effect of cells of the immune system largely depends on the induction of apoptosis in the tumour cells. Therefore, disruption of the apoptosis pathway is, besides loss of antigen presentation or local or systemic immune suppressive factors, a possible important cause for resistance to immune therapy.

3. Disruption of the intrinsic apoptosis pathway may explain resistance to apoptosis but is not related to a poor clinical outcome

CTLs are thought to kill their target cells via induction of apoptosis. As indicated in the introduction CTLs and NK cells can induce apoptosis via different, partly overlapping pathways (see figure 3 in the introduction). Because our data presented in this thesis suggest that a properly functioning cellular immune response determines favourable clinical outcome, it was expected that melanoma cells in these clinically favourable cases are sensitive to the cell death inducing effect of CTLs and NK cells and thus should have a more or less intact apoptosis pathway. We were able to isolate melanoma cells from dissected tumour material for mRNA profiling and therefore the apoptosis pathway was investigated in considerable detail.

We observed that disruption of the downstream convergence apoptosis pathway is a frequent event in melanomas. This same phenomenon was also detected in aggressive lymphomas in which disruption of this pathway resulted in resistance to chemotherapy and poor clinical outcome. However, in melanoma patients we found no correlation between disruption of this intrinsic apoptosis pathway and poor clinical outcome. The patients were treated with the ASI vaccination protocol and favourable outcome was expected to result from cellular immune response and our data show that sensitivity is apparently not dependent of activation of this intrinsic pathway.

A known inhibitor of the extrinsic pathway is c-FLIP.[20] On cytospins from different melanoma cell lines c-FLIP expression was immuno histochemically detected, using the validated methods described in **chapter 5**, and c-FLIP expression was confirmed on western Blot. However, no expression of c-FLIP could be detected in paraffin embedded tissue of melanoma biopsies, even when different antibodies and different staining protocols were used (addendum). When tested on the mRNA level, relatively low levels of c-$FLIP_{long}$ and c-$FLIP_{short}$ were detected. Probably, c-FLIP expression levels in paraffin embedded tissue of melanoma biopsies is below the detection limit. Thus, the role of c-

FLIP expression in melanoma cells in resistance to immune system induced tumour cell death needs to be further evaluated.

Future directions

The association of the cellular immune response with tumour progression or clinical outcome, described in this thesis, gives us insight in possibilities to conquer resistance to immune therapy in melanoma patients. Based on our results, we expect that enhancing the effectiveness of the cellular immune response against melanoma cells improves clinical outcome in melanoma patients. Furthermore, restoring sensitivity of melanoma cells to this cellular immune response might improve outcome in patients with disseminated melanoma.

Lack of an effective immune response appears to be, at least partly caused by loss of antigen presentation in the context of the appropriate MHC class I molecules. However re-establishment of appropriate antigen presentation cannot be achieved easily[21] but therefore it might be more worthwhile to enhance the cellular immune response by increasing MHC class I expression on tumour cells.
Another way to enhance the effectiveness of the cellular immune response might be by restoring sensitivity to GrB induced apoptosis, by interfering with the function of PI-9 [22] using small molecule antagonists. However, such antagonists are as yet not available.

In this thesis we also observed that the intrinsic apoptosis pathway is constitutively activated in approximately 50% of metastasised melanomas with concomitant downstream disruption of this pathway. One of the proteins that may cause this downstream inhibition is XIAP. Neutralising the apoptosis inhibiting function of XIAP should result in spontaneous induction of apoptosis. Such XIAP neutralizing antagonists are currently available[23,24] and we have shown previously that this XIAP antagonist indeed results in spontaneous apoptosis in lymphoma cells with constitutive activation of the intrinsic apoptosis pathway.

Concluding remarks

The cellular immune response as reflected by the presence of activated (i.e. GrB^+) TILs in the primary tumour is an important prognostic factor in melanoma patients. Therefore, immune therapy aiming at boosting the effectiveness of the cellular immune response and/or restoring the sensitivity of melanoma cells to this cellular immune response might protect patients for further metastasis and improve outcome in patients with disseminated melanoma. Finally, neutralizing downstream inhibition of the intrinsic apoptosis pathway might be an alternative treatment for those patients in which the melanoma cells demonstrate constitutive caspase 9 activation.

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