On the biological relevance of MHC class II and B7 expression by tumour cells in melanoma metastases

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A large number of studies have indicated that specific immune reactivity plays a crucial role in the control of malignant melanoma. In this context, expression of MHC I, MHC II and B7 molecules by melanoma cells is seen as relevant for the immune response against the tumour. For a better understanding of the biological relevance of MHC II and B7 expression by tumour cells in metastatic melanoma, we studied the expression of these molecules in melanoma metastases in relation to the inflammatory response, regression of the tumour and survival from 27 patients treated with biochemotherapy (30 mg m⁻² Cisplatin and 250 mg m⁻² decarbazine (dimethyl-triazene-imidazole-carboxamide, DTIC) on days 1–3 i.v., and 10⁷ IU IFN-α2b 3 days a week s.c., q. 28d). In 19 out of 27 lesions studied, we found expression of MHC II by the tumour cells, while only in one out of 11 tumour biopsies obtained from untreated metastatic melanoma patients, MHC II expression was detected. Expression of B7.1 and B7.2 by tumour cells was found in nine out of 24 and 19 out of 24 lesions, respectively. In all cases where B7.1 expression was found, expression of B7.2 by the tumour cells was also seen. In general, no or only few inflammatory cells positive for B7 were found. Expression of MHC II by tumour cells was positively correlated with the presence of tumour-infiltrating lymphocytes, regression of the tumour, and with time to progression (TTP) and overall survival (OS) of the patient. However, no significant correlation between B7.1 or B7.2 expression and regression of the tumour, TTP or OS was found. In light of other recent findings, these data altogether do support a role as biomarker for MHC II expression by tumour cells; however, its exact immunological pathomechanism(s) remain to be established.

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CD4+ cells, presenting endogenous tumour antigens (Chen et al., 1994; Pulaski and Ostrand-Rosenberg, 1998; Ostrand-Rosenberg et al., 1999). In human primary melanoma, however, expression of MHC class II by tumour cells is associated with an unfavourable prognosis (van Duinen et al., 1988; Ruiter et al., 1991). In addition, Becker et al. (1993) reported that autologous melanoma cell lines render CD4+ T cells unresponsive to subsequent stimulation in an MHC class II restricted manner. This effect could be abrogated by transfection of the melanoma cells with the costimulatory molecule B7 (Becker et al., 1993). In summary, while studies in some animal tumour models suggest that MHC II expression by tumour cells increases the immunogenicity of the tumour, data on human tumour cells suggest that MHC II expression might reduce the immunogenicity of the tumour in patients with melanoma metastases.

In this paper, we address the relevance of expression of MHC II and the costimulatory molecules B7.1 and B7.2 in relation to response to treatment with biochemotherapy.

MATERIALS AND METHODS

Patient data

The present study describes 27 patients (18 males and nine females) with metastatic malignant melanoma, 15 with regional disease and 12 with resectable systemic disease. The median age was 58 (range 34–71) years and their Karnofsky status was 70 or more. Recurrences were cytologically verified by fine-needle aspirates before treatment was started. One metastasis from each patient was studied. In patients with systemic disease, easily resectable metastases were chosen. In patients with regional disease, non-necrotic biopsies were randomly chosen by the pathologist. In addition, tumour biopsies obtained from 11 untreated metastatic melanoma patients (nine males, two females) were studied for MHC II expression. All these patients had regional disease and a median age of 57 (range 39–79) years. Recurrences were cytologically verified by fine-needle aspirates. One metastasis from each patient was studied.

The study was approved by the ethical committee at the University Hospital of Linköping, Sweden.

Pretreatment investigations and treatment schedule

Pretreatment investigations were as described previously (Häkansson et al., 2001). Treatment consisted of 30 mg/m² cisplatin (cis-diamine-dichloro-platinum) and 250 mg/m² dacarbazine (dimethyl-triazene-imidazoly-carboxamide, DTIC) on days 1–3 i.v., and 10³ IU IFN-α2b 3 days a week s.c. The duration of one cycle was 28 days.

Preparation of tumour biopsies and immunological staining of tissue sections

Biopsies from the resected metastases were immediately snap frozen and stored at −70°C until further use. Tissue sections, 6–7 μm thick, were fixed with phosphate-buffered 4% paraformaldehyde, pH 7.4 (Riedel-de Haen AG, Seelze, Germany) supplemented with 5.4 g l⁻¹ of glucose for 5 min and then washed three times in Hanks’ balanced salt solution (BSS, Gibco, Paisley, UK), supplemented with 0.01 M Hepes solution before staining. For detection of the expression of MHC antigens, B7.1, B7.2 and the presence of T-cell subsets, the following monoclonal antibodies were used: anti-HLA, Stockholm, Sweden, DR for MHC I (0.57 μg ml⁻¹, clone L243; Becton Dickinson), anti-HLA-ABC for MHC I (0.57 μg ml⁻¹, clone W6/32; Dakopatts, Oxon, UK), anti-B7.1 (20 μg ml⁻¹, clone 37711.11; R&D systems), anti-B7.2 (20 μg ml⁻¹, clone 37301.11; R&D systems), anti-CD3 (7.5 μg ml⁻¹, clone UCHT1; Dako), Leu-3a for CD4 (1 μg ml⁻¹; Becton Dickinson) and Leu-2a for CD8 (0.25 μg ml⁻¹; Becton Dickinson). Sections for T-cell subsets were blocked with normal rabbit serum before the first staining. Sections for MHC I and MHC II were blocked with normal goat serum and sections for B7.1 and B7.2 with 10% AB serum. Tissue sections were then incubated with primary antibodies for 30 min or overnight (B7.1 and B7.2) at room temperature. Mouse IgG1 (Dakopatts, Sweden) was used as a negative control for the subsets of inflammatory cells and the B7 antibodies. Mouse IgG (Sigma, Stockholm, Sweden) was used as a negative control for the major histocompatibility antigens. After the slides were washed in BSS-saponin, biotinylated rabbit-anti-mouse immunoglobulin was added at 1:100 dilution in BSS-saponin for anti-CD3, anti-CD4 and anti-CD8. They were then incubated with peroxidase-labelled streptavidin (P0397, DAKO, Stockholm, Sweden) at 1:100 dilution in BSS-saponin for 30 min. DAB (3,3′-diaminobenzidine, D-5637, Sigma, Stockholm, Sweden) was used as a substrate. Sections stained for MHC I and MHC II were washed in BSS-saponin; then, goat-anti-mouse immunoglobulin was added at 1:25 dilution in BSS-saponin and left to incubate for 30 min in a moist chamber. The slides were then washed in BSS-saponin, and mouse APAAP complex diluted at 1:25 in BSS-saponin was added and incubated for 30 min. For B7.1 and B7.2, the Envision system was used as the second step (DAKO, Stockholm, Sweden). All antibody solutions containing 2% normal blood donor AB serum.

After washes in BSS-saponin and TBS, the Fast-red substrate supplemented with levamisole to block endogenous alkaline phosphatase was added to the sections (MHC and B7) and left to incubate for 20 min at room temperature. The slides were then washed in TBS, pH 7.6, counterstained in Mayer’s haematoxylin for 15 min and mounted in Glycergel (Dakopatts, Sweden).

Evaluation of tumour regression and histopathological criteria

The occurrence of tumour regression was evaluated by histopathological examination of tumour biopsies based on the description of regressive changes in malignant melanoma as previously described (McGovern, 1975; Kang et al., 1993; Häkansson et al., 1996, 1998). The following criteria of tumour regression were used in this study: (1) low and variable density of tumour cells, particularly variation in density within the same tumour nodule; (2) disorganisation of the architecture of the tumour with nests of remaining tumour cells surrounded by stromal tissue and (3) fibrosis. The mononuclear infiltrate was not used as a criterion of histopathological tumour regression in this study. The regressive changes were scored on control sections not stained for mononuclear cells. The signs of regression vary from no signs to almost complete destruction with only a few tumour cells present. The degree of tumour regression was scored semiquantitatively as less than 10%, comprising 10–25, 25–50, 50–75 or 75–100% of the section area. A score of >25% was generally considered significant unless otherwise stated. The scoring was performed independently by two observers (MRB, BG) and consensus was reached for all scorings.

Statistical analysis

Survival curves were plotted using the Kaplan–Meier method. Differences in clinical response in terms of time to progression and OS between patients showing differences in expression of MHC II or B7 by the tumour or differences in regressive changes in the tumour were analysed using the log rank test. A two-sided Fisher’s exact test was used to analyse whether or not an increased level of MHC II expression coincided with an increased level of histological regression. Calculations of significance were performed using Graph Pad Prism version 3.02 for Windows (Graph Pad Software, San Diego, CA, USA).

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RESULTS

MHC I and MHC II expression in metastatic lesions from treated and untreated melanoma patients

The expression of MHC classes I and II was studied in 27 lesions from biochemotherapy-treated patients by immunohistochemical analysis. All lesions were homogeneously positive for MHC I. In contrast, a heterogeneous expression of MHC II was found between and within lesions. In eight out of these 27 lesions, the tumour cells were negative for MHC II, while in the remaining 19 lesions, 10 to more than 75% of the tumour cells were positive for MHC II (Figure 1A). In contrast, in 11 tumour biopsies derived from untreated patients, only in one out of the 11 MHC II expression was detected in approximately 75% of the tumour cells. In all cases, MHC II expression of the tumour cells was associated with a pronounced presence of TIL as exemplified in Figure 2A and B. In all cases, these TIL consisted of CD4+ as well as CD8+ cells. There was some heterogeneity in the relative occurrence of these subpopulations, but this heterogeneity could not be linked to whether or not adjacent tumour cells were positive for MHC II. In all cases, the majority of inflammatory cells were MHC II-positive.

![Figure 1](image1.png)  
**Figure 1**: Distribution of MHC II (A) and B7 (B) expression over the lesions studied.

![Figure 2](image2.png)  
**Figure 2**: Expression of MHC II (A: red-stained cells, black arrows indicating positive tumour cells and white arrows indicating positive inflammatory cells) and presence of CD3+ cells (B: brown-stained cells; the brown staining is not melanin) in corresponding areas of a metastatic lesion. Expression of B7.1 (C: black arrows indicating positive-staining tumour cells and white arrows indicating negative inflammatory cells) and B7.2 (D: black arrows indicating positive tumour cells and white arrows indicating positive inflammatory cells) in corresponding areas of a metastatic lesion (red-stained cells).
B7 expression in metastatic lesions of treated melanoma patients

Also for B7, heterogeneous expression between and within lesions was found. Out of 24 lesions studied for B7.1 and B7.2 expression, 15 were negative for B7.1 and five for B7.2 (Figure 1B). In most cases where B7 expression was detected, the staining intensity of the tumour cells was weak as compared to the staining intensity of inflammatory cells when positive (Figure 2C). For B7.1 in none of the 24 lesions, positive-staining inflammatory cells were observed. In contrast, for B7.2 in 17 out of 24 lesions, positive staining of inflammatory cells was seen (Figure 2C and D). In general, however, only a limited fraction of the inflammatory cells were positive for B7.2. In all lesions where B7.1 positive tumour cells were found, positive staining for B7.2 was also found. In Figure 3 the relative distribution of B7.1 and B7.2 positive tumours, that is more than 10% of the tumour cells positive, in relation to the expression of MHC II by tumour cells is shown. Although it seems that B7.1 expression is found more frequently in tumours that are significantly positive for MHC II, that is more than 25% of the tumour cells positive for MHC II, no specific relation between MHC II expression and B7.1 or B7.2 expression could be discerned.

Relation between MHC II and B7 expression by tumour cells and the occurrence of histological regression in metastatic lesions of melanoma patients

Histological regression of tumour lesions was scored according to criteria as published before (McGovern, 1975; Kang et al., 1996, 1998) and as specified under Materials and Methods. We plotted the extent of histological regression against the percentage of MHC II-positive tumour cells found in each lesion (Figure 4A). In 18 out of 27 lesions studied, regressive changes were observed. Out of these 18, 10 were significantly positive for MHC II, that is more than 25% of the tumour cells, while in the remaining nine lesions that showed no significant regressive changes, that is less than 25% of the section area, only in one out of nine lesions MHC II expression in more than 25% of the tumour cells was detected. This difference was statistically significant (P = 0.042; two-sided Fisher’s exact test).

Similar to MHC II, we plotted the extent of histological regression against the percentage of B7-positive tumour cells found in each lesion (Figure 4B and C). There was, however, no apparent relation between the occurrence of regressive changes and B7 expression by the tumour cells.

Relevance of MHC II and B7 expression by tumour cells to clinical response parameters

In order to establish whether or not expression of MHC II and or B7 by tumour cells might be of clinical relevance, we performed survival analysis on patients in relation to the extent to which their tumour expressed MHC II or B7. For TTP as well as OS, there was a significant trend for increased survival in relation to increased expression of MHC II by the tumour (P = 0.034 and 0.043, respectively; log rank test for trend). The use of a cutoff point of 25% of the tumour cells positive for MHC II results in a significant difference in TTP and OS for the two resulting patient groups (P = 0.034 and 0.032, respectively; Figure 5).

A similar approach was used for the relation between B7 expression by the tumour and TTP and OS of the patients. No significant relation between B7 expression by the tumour and TTP or OS was found (Figure 6).

DISCUSSION

Metastatic malignant melanoma is, despite various treatment strategies, still associated with a poor prognosis. In other studies, we have demonstrated that treatment with IFNα or IFNγ in combination with cisplatin and DTIC results in early (incomplete) regression of metastases (Håkansson et al., 1996, 2001). These responses showed a strong correlation with the amount of tumour-infiltrating CD4+ lymphocytes present before treatment. However, objective remission following IFNα-based treatment is still only 15% (Legha, 1997). These observations suggest that an initial effective antitumour immune response is initiated following treatment, but that shortly thereafter immunological resistance occurs (Håkansson et al., 1999, 2001).

Given the apparent important role of CD4+ cells in the induction of an effective immune response to the tumour, we studied the expression of MHC II and B7 in melanoma metastases of patients treated with IFNα, cisplatin and DTIC, in relation to the
inflammatory response, regression of the tumour and survival of the patients. In 19 out of 27 lesions studied, we found expression of MHC II by the tumour cells. In contrast, in 11 tumour biopsies obtained from metastatic melanoma patients before treatment, only in one lesion MHC II expression by the tumour cells was seen. These findings suggest a treatment-related expression of MHC II by the tumour. This is further supported by the findings in three fine-needle aspirate biopsies derived from three patients before treatment (data not shown). In none of the three aspirates positive staining of the tumour cells for MHC II was obtained, while in two of the three corresponding post-treatment tumour biopsies MHC II-positive tumour cells were detected (25–50 and 75–100%, respectively). As also found by others (Bröcker et al, 1984, 1988; Guerry et al, 1987; Hersey and Jamal, 1990), we found a close correlation between MHC II expression and presence of TIL. There was also a positive correlation between MHC II expression by tumour cells and regression of the lesion. MHC II expression was also positively correlated with TTP and OS of the patients. Patients whose tumours showed MHC II-positive staining of more than 25% of the tumour cells had a significant longer TTP and OS survival than those whose tumour was negative or only limited positive (less than 25% of the tumour cells) for MHC II. In this study, MHC II expression on tumour cells thus represents a biomarker for response. This expression of MHC II by tumour cells might be of particular importance with regard to the observation that the occurrence of CD4+ lymphocytes before initiation of IFN-α treatment or biochemotherapy was significantly correlated to therapeutic response (Håkansson et al, 1996, 2001). During IFN-α treatment, these lymphocytes were also shown to migrate from the stromal areas surrounding the tumour nodules.

Figure 5  Kaplan–Meier analysis for TTP and OS on patients whose tumour showed significant levels, that is > 25% of the tumour cells, of MHC II expression.

Figure 6  Kaplan–Meier analysis for TTP and OS on patients whose tumour showed different levels of B7.1 and B7.2 expression.
into these nodules close to the tumour cells. Related to this redistribution of the lymphocytes regressive tumour changes appeared. These findings are highly suggestive of an antitumour activity exerted by CD4+ lymphocytes. A prerequisite for an antitumour activity by these lymphocytes is, however, that the tumour cells express MHC II (Zennadi et al, 2001).

It is conceivable that MHC II expression by the tumour cells is a result of inflammatory regression of the tumour following treatment. In this inflammatory milieu IFN-γ most likely will be produced, resulting in the local induction of MHC II expression by tumour cells, which would be in accordance with the data presented in this paper. However, it remains arguable whether the MHC II-positive tumour cells have a local stimulatory effect on tumour infiltrating CD4+ cells or a suppressive effect or no effect at all. In vitro studies have shown that binding of CD4+ cells to MHC II in the absence of B7 molecules can lead to an immune suppressed or anergic state of the CD4+ cell (Becker et al, 1993). MHC II expression by nonprofessional APC is also considered a negative feedback mechanism to maintain peripheral tolerance (Marelli-Berg and Lechler, 1999). In this light, MHC II expression by tumour cells could represent a negative factor in the immunological control of cancer and may be an explanation for the finding that MHC II expression in primary melanoma and locoregional melanoma metastases is associated with an unsatisfactory prognosis (van Duinen et al, 1988; Ruiter et al, 1991). In the present study, however, MHC II expression by tumour cells in metastatic lesions from biochemotherapy-treated patients was associated with longer TTP and OS. As shown in this study, in the majority of MHC II-positive lesions also expression of B7.1 and/or B7.2 by tumour cells was found. Possibly, this coexpression of MHC II and B7 increases the immunogenicity of the tumour as has been demonstrated in in vitro studies and mouse tumour models (Becker et al, 1993; Chen et al, 1994; Baskar et al, 1996; Pulaski and Ostrand-Rosenberg, 1998; Ostrand-Rosenberg et al, 1999). Surprisingly, however, no significant correlation between B7.1 or B7.2 expression in itself and regression of the tumour, TTP or OS was found. In any case, the question as to why in most patients the immunological control of tumour growth is only partial still remains. In this respect the results presented in this paper could also be seen in a different light. As mentioned earlier, it is conceivable that MHC II expression by the tumour cells, as frequently observed in the metastatic lesions of biochemotherapy-treated patients, is a result of the local inflammatory milieu following immune reactivity against the tumour, thus indicating the successful (systemic) induction of an antitumour immune response, and hence associated with regressive changes in the lesions and longer TTP and OS of the patients. Locally, the expression of MHC II on tumour cells may still have a detrimental effect on the T-cell response by causing local downregulation or anergy of the T cells. Possibly, this might influence the expression of CD28 and the ζ-chain, as these molecules were found to be downregulated, in particular, in regressive tumour areas during IFN-γ treatment or biochemotherapy (Håkansson et al, 1999). In this respect, data on the heterogeneous effects of B7 molecules on T-cell modulation may also be relevant (Martin-Fontecha et al, 1996; Kosmaczewska et al, 2001). Both B7.1 and B7.2 bind to CD28 and CTLA4, but with different affinity and possibly resulting in different signalling pathways (Martin-Fontecha et al, 1996; Kosmaczewska et al, 2001). For instance, while CD28 triggering has been shown to be crucial in T-cell activation, CTLA4 triggering appears to be only associated with downregulation of T-cell responses (Greenfield et al, 1998; Kosmaczewska et al, 2001). Also the role of other costimulatory molecules may be of significance. Although B7 molecules are often seen as crucial factors for costimulation, some reports have also indicated that other costimulatory molecules, for example, ICAM-1, may be sufficient for efficient T-cell activation (Robinson and Delvig, 2002).

Other recent findings, however, may also cast a different light on the immunological significance of MHC II expression by tumour cells. The relevance of tumour cells as APC for CD4+ cells appears to be dependent on the coexpression of MHC II and accessory molecules such as the invariant chain (II) and HLA-DM (Armstrong et al, 1997; Brocke et al, 2002), each of which are under the primary control of CIITA, the Class II transactivator. Normally MHC II is expressed in concert with these accessory molecules and mainly exogenous antigens are presented (Masternak et al, 2000; Robinson and Delvig, 2002). In this situation, tumour cells appear to be poor immunogens (Armstrong et al, 1997; Blanck, 1999). However, when MHC II is expressed without coexpression of II and HLA-DM, such as in MHC II-transduced or -transfected tumour cells, they may become potent immunogens by presenting endogenous antigens, provided that B7 expression is induced following MHC II engagement or expressed through cotransfection or cotransduction of the B7 gene(s) (Baskar et al, 1996; Armstrong et al, 1997). MHC II-expressing tumour cells in tumours may thus be poor APC or even immunologically inert to CD4+ cells. The question then remains as to why in certain cases MHC II expression by tumour cells is associated with a poor prognosis in melanoma and why in other cases, as in the present study, MHC II expression is associated with an apparently more favourable prognosis (van Duinen et al, 1999). In this respect, the results presented in this paper could also be seen in a different light. As mentioned earlier, it is conceivable that MHC II expression by the tumour cells, as frequently observed in the metastatic lesions of biochemotherapy-treated patients, is a result of the local inflammatory milieu following immune reactivity against the tumour, thus indicating the successful (systemic) induction of an antitumour immune response, and hence associated with regressive changes in the lesions and longer TTP and OS of the patients. Locally, the expression of MHC II on tumour cells may still have a detrimental effect on the T-cell response by causing local downregulation or anergy of the T cells. Possibly, this might influence the expression of CD28 and the ζ-chain, as these molecules were found to be downregulated, in particular, in regressive tumour areas during IFN-γ treatment or biochemotherapy (Håkansson et al, 1999). In this respect, data on the heterogeneous effects of B7 molecules on T-cell modulation may also be relevant (Martin-Fontecha et al, 1996; Kosmaczewska et al, 2001). Both B7.1 and B7.2 bind to CD28 and CTLA4, but with different affinity and possibly resulting in different signalling pathways (Martin-Fontecha et al, 1996; Kosmaczewska et al, 2001). For instance, while CD28 triggering has been shown to be crucial in T-cell activation, CTLA4 triggering appears to be only associated with downregulation of T-cell responses (Greenfield et al, 1998; Kosmaczewska et al, 2001). Also the role of other costimulatory molecules may be of significance. Although B7 molecules are often seen as crucial factors for costimulation, some reports have also indicated that other costimulatory molecules, for example, ICAM-1, may be sufficient for efficient T-cell activation (Robinson and Delvig, 2002).
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