Control of T-Cell–Mediated Immune Response by HLA Class I in Human Pancreatic Carcinoma

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ABSTRACT

Purpose: Cell surface HLA class I molecules present peptides derived from human cellular proteins to T cells. In the present study, we investigated the expression of HLA class I in human pancreatic carcinoma.

Experimental Design: The expression of HLA class I antigen and the extent of tumor infiltration by T cells were investigated in 46 primary tumors and in 14 metastases of pancreatic cancer by standard immunohistochemistry.

Results: The locus-specific expression of HLA I was reduced in 61% of primary tumors and in 93% of metastases. The total loss of this molecule complex was detected in 6% of primary tumors and in 43% of metastases. Pancreatic carcinoma and peritumoral tissue showed a significantly higher infiltration by CD3+, CD4+, and CD8+ T-cells compared with the tumor-distant pancreatic tissue. The negative expression of HLA class I was uniformly accompanied by a low density of tumor-infiltrating cytotoxic T-cells whereas the HLA class I–positive tumors were characterized by a substantial lymphocyte accumulation. However, the infiltration by cytotoxic T-cells was not correlated with the density of tumor cells. Patients with a high accumulation of cytotoxic cells showed a longer median survival.

Conclusions: Pancreatic carcinoma frequently induces a cellular immune response that results in intratumoral and peritumoral T-cell infiltration. The expression of HLA class I is frequently lost in pancreatic carcinoma, which represents an effective mechanism to escape the tumor infiltration by cytotoxic T-cells. However, the infiltration by cytotoxic cells represents a favorable prognostic sign in pancreatic cancer patients.

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INTRODUCTION

The genetic loci involved in rejection of foreign or virally infected tissue forms are known as MHC or as HLA (1). Cell surface MHC molecules present peptides derived from intracellular and extracellular proteins to T cells, which are able to recognize these cell-bound antigens only in association with the MHC complex. Two classes of MHC, class I and class II, are known that act as guidance systems for T cells. The mechanism of antigen presentation and recognition by T cells is well known. The proteins produced endogenously are cut by proteosomes in the cytosol, transported to the cellular membrane by peptide transporters, and presented by MHC class I. MHC class II binds mainly exogenous peptides (1). In the normal situation, all peptides are derived from self cellular proteins, which are tolerated by self T-cells. If the cells are mutated or infected with virus, foreign peptides are produced and presented in addition to the self peptides on MHC class I. Cytotoxic T-cells then bind to MHC class I by the TCR/CD8 receptor complex (2). They recognize these peptides as foreign antigens and destroy selectively those cells. This system of peptide presentation by MHC class I and recognition of self or non-self antigens by T cells provides an effective protection of the organism from neoplastic and infected cells.

The development of malignant tumors besides neoplastic transformation represents the failure of host resistance to eliminate aberrant cells. Neoplastic cells frequently express surface antigens, which can be recognized as foreign and activate the cytotoxic reaction of T cells. However, effective rejection of neoplastic cells requires expression of MHC class I associated with foreign peptides. Previous investigations showed that the expression of HLA class I in various human malignant tumors is markedly altered (3–6). An aberrant expression of MHC class I has been proposed as a major mechanism to escape the effective T-cell response against malignant cells (4, 7).

The present investigation was carried out to study the linkage of cellular immune response by HLA class I expression in patients with pancreatic carcinoma. We could show by immunohistochemistry that the expression of HLA class I is frequently altered in pancreatic carcinoma. Only pancreatic carcinomas which showed a positive or partially positive expression of HLA class I were accompanied by profound lymphocyte infiltration of tumor tissue. HLA class I–negative tumors showed a low density of tumor-infiltrating cytotoxic T-cells. Patients with a high accumulation of cytotoxic cells showed a longer median survival.

MATERIALS AND METHODS

Patients and Tissue Samples. Patients admitted to the study were undergoing surgery for pancreatic carcinoma at the Department of Surgery at the University of Heidelberg. In all cases the protocol was approved by the local ethical committee and informed consent according to the Helsinki Declaration was obtained. Sixty tissue samples of ductal pancreatic carcinoma (46 primary tumors and 14 metastases from different patients)
were collected. Eleven specimens of primary tumors contained both tumor and peritumoral tissue. One additional sample of the primary tumor and a metastasis from the same patient were investigated. All tumors were histopathologically classified as well differentiated, moderately differentiated, and poorly differentiated adenocarcinomas according to WHO classification. Additionally, 12 samples of nonmalignant pancreas were obtained 5 to 10 cm away from the pancreatic carcinoma. These specimens were determined as tumor-distant tissue in contrast to tumor-surrounding pancreatic (e.g., peritumoral) tissue. Two specimens of the spleen were used as control. Each sample was snap frozen and stored in liquid nitrogen. The clinical information about operated patients was prospectively documented for further statistical analysis. For survival analysis, only R0-resected patients were selected (n = 24).

**Immunohistochemical Staining.** Sections of 5 μm were cut, air dried, and fixed in acetone. The slides were stored at −20°C until further use. The sections were stained by indirect three-step immunohistochemistry using the LSAB-kit (DAKO GmbH, Hamburg, Germany) and counterstained using Mayer’s hemalum (Fluka, Steinheim, Germany). The following clones of purified monoclonal antibodies were used: W6/32 (against monomorphic epitope of HLA class I antigen), 246-E8E7 (anti-β2-microglobulin), 108-2C5 (subset of HLA-A locus-encoded gene products), JOAN-1 (HLA-B locus-encoded gene products), CATA-1 (anti-HLA-A25 and HLA-w32), AE1/A3 (pan-cytokeratin), UCHT1 (anti-CD3, T-cells), MT310 (anti-CD4), DK25 (anti-CD8), UCHL1 (anti-CD45R0, memory T-cells), and MOCA1 (anti-CD56, natural killer cells). The clones 108-2C5, JOAN-1, CATA-1, and 246-E8E7 were obtained from NeoMarkers (Fremont, CA). All other antibodies were purchased from DAKO.

The expression of HLA class I and β2-microglobulin was assessed by two investigators (E.R. and F.A.) unaware of the status of other immunohistologic and clinical data. The extent of expression by tumor cells was investigated using a scale as described by Ramal et al. (8): negative, <25%; heterogeneous, 25% to 80%; positive, >80% of tumor cells.

The quantitative analysis of immunohistochemical staining was done by computer-assisted image analysis. For this aim, the microscopic fields were randomly chosen by light microscope, digitalized by a color video camera to histologic images, and saved on a computer. All measurements were done using a special software (Histo, Department of Experimental Surgery, University of Heidelberg, Heidelberg, Germany). This software allows us to separate the areas expressing different colors and to measure the surface area of these separated regions. The relative fraction of tumor cells (versus stroma) was assessed by low magnification (×50) on a histologic field of 2.9 mm² and was expressed as percentage of cell surface positively stained by anticytokeratin antibodies.

For the measurement of lymphocyte density, six fields (field surface, 0.12 mm²) were randomly chosen by a high magnification (×250). The number of positively stained lymphocytes was obtained and expressed per square millimeter of tumor surface.

**Statistical Analysis.** Statistical analysis was done using SPSS software (Version 11.5.1, SPSS Inc., Chicago, IL). The distribution of lymphocyte density was presented by box-and-whisker plots and dot plots. Subgroups of patients were compared with respect to lymphocyte density using the Mann-Whitney U-test, the Wilcoxon’s sign rank test, and the Kruskal-Wallis test, if appropriate. Spearman correlation coefficient was used to determine the association between lymphocyte infiltration and value of the cytokeratin staining. The expression of HLA class I was compared with tumor differentiation and tumor presentation by Fisher’s exact test. Kaplan-Meier estimation was used to analyze the survival of pancreatic carcinoma measured from the date of surgery. The patients alive at last follow-up were censored. Median survival with 95% confidence interval (CI) was given. The associations among negative versus heterogeneous/positive expression of HLA class I, lymphocyte infiltration, and survival were examined by the log-rank test. Statistical significance was assumed at P ≤ 0.05. All tests used were two sided.

**RESULTS**

Expression of HLA Class I. The expression of HLA class I in nonmalignant and pancreatic cancer tissue was investigated using antibodies against the monomorphic determinant of this molecule. The control tissue of the spleen showed a high level of HLA class I expression by any cell type except the vascular smooth muscle cells. Nonmalignant pancreas expressed HLA class I heterogeneously. Ductal, endothelial, and endocrine cells showed an abundantly positive expression of HLA class I (Fig. 1A). The tumor-distant exocrine tissue did not express this molecule complex. The expression of HLA class I in pancreatic cancer tissue was detected on stromal, endothelial, and lymphoid cells. Tumor cells expressed this molecule in a nonuniform fashion. The stainings with W6/32 and anti-β2-microglobulin antibodies showed an identical expression. The results are summarized in Table 1. The staining of HLA class I by locus-specific antibodies and clone CATA-1 showed further alterations of HLA class I expression from positive to heterogeneous/negative expression and from heterogeneous to negative expression (Table 1). The loss of HLA class I was significantly higher in metastases than in primary tumors (P < 0.001) (Table 1). Analysis of HLA class I in primary tumor and metastasis of the same patient showed the reduction from positive to heterogeneous expression by three antibodies (W6/32, 246-E8E7, and 108-2C5) and from heterogeneous to negative expression by other clones (JOAN-1 and CATA-1). Negative expression of HLA class I was more frequently found in the poorly differentiated compared with the well differentiated or moderately differentiated tumors (Table 2). However, this difference was not statistically significant (P > 0.05, Fisher’s exact test).

Lymphocyte Infiltration. Tumor-distant pancreatic tissue included few distributed lymphocytes which showed both CD3+ and CD8+ markers (Fig. 1B). The CD4+ and CD56+ lymphocytes in tumor-distant pancreatic tissue were nearly absent. In contrast to tumor-distant nonmalignant tissue, pancreatic carcinoma and tumor-surrounding pancreatic tissue frequently showed a high infiltration by CD3+, CD4+, and CD8+ lymphocytes. The lymphocyte distribution throughout the tumor tissue was heterogeneous. Lymphocytes infiltrated tumor
The mean and maximal densities of CD3+ and CD4+ T-cells in primary tumors were significantly higher compared with the tumor-distant pancreas (Fig. 3A and B), whereas the mean lymphocyte density of CD4+ lymphocytes was not significantly different (Fig. 3A). The mean and maximal densities of CD3+ T-cells were significantly higher in tumor metastases than in tumor-distant pancreas; there were no other significant differences between metastases and normal tissue. Peritumoral tissue showed a dense infiltration by CD3+, CD4+, and C8+ T-cells, which was significantly higher than in primary tumors, metastases, and tumor-distant tissue (Fig. 3A and B). The infiltration by CD45R0+ T-cells in tumor tissue corresponded well to the infiltration by CD8+ lymphocytes. The infiltration by natural killer cells of malignant and nonmalignant tissues was very low.

The analysis of lymphocyte density at different expressions of HLA class I showed that the HLA class I–negative tumor showed a low infiltration with T cells, which did not exceed 200 lymphocytes/mm² (Figs. 1E and F and 4). In contrast to these tumors, pancreatic carcinomas with a positive or partially positive (heterogeneous) expression of HLA class I showed a significantly higher maximal lymphocyte infiltration by CD4+ and CD8+ T-cells than HLA class I–negative tumors (P = 0.02 and P < 0.001, respectively), which exceeded 200 cells/mm² in most cases (Figs. 1 and 4).

**Influence of Lymphocyte Infiltration on Tumor Cell Density and Patient Overall Survival Time.** Because of T-lymphocytes being able to destroy malignant tumor cells, we evaluated the correlation between these cells and the fraction of tumor cells in all patients. The fraction of malignant cells was assessed by an anti-cytokeratin staining of all epithelial cells. The mean surface of positive staining with cytokeratin was 22.1 ± 13.1% in primary tumors and 30.8 ± 15.2% in metastases. Statistical analysis showed no correlation between the value of cytokeratin-positive surface and the mean (r = −0.20) as well as the maximal (r = −0.18) densities of lymphocyte infiltration with CD8+ T-cells.

The analysis of the survival time was done in all patients with available follow-up data after R0 resection (n = 24) and after palliative/diagnostic operations (n = 12). Median survival time after R0 resection was 10 months (95% CI: 7-12 months) whereas patients after palliative/diagnostic operations had a median survival of 3 months only (95% CI: 2-4 months) (P < 0.001). Kaplan-Meier analysis was done to compare the expression of HLA class I and that of tumor infiltration by cytotoxic cells on survival time after R0 resection. The highest values of the mean (65 cells/mm²) and maximal
(180 cells/mm²) lymphocyte densities in normal pancreatic tissue were used as a threshold value to compare the survival time and the density of CD8+ lymphocytes in cancer tissue. The median, CI, and significance values are summarized in Fig. 5. Six patients were alive at last follow-up. Although statistical analysis showed no significant difference in survival time distribution between the analyzed groups (Fig. 5), the median survival of patients with high density of CD8+ T-cells
DISCUSSION

The development of most solid tumors is associated with a reduced or negative expression of HLA class I (7). This molecule complex mediates interaction with T-lymphocytes, which recognize peptides bound to class I molecules, and natural killer cells, which recognize particular allelic forms of the class I complex. Previous investigations showed that colorectal (9, 10), breast (11), cervical (5), head and neck carcinoma (3), and melanoma (12) showed frequent alterations of this important molecule complex. Different mechanisms such as loss of heterozygosity (9), loss of β2-microglobulin (10), transporter proteins (13, 14), and methylation of DNA (15) have been reported to be responsible for the altered expression of HLA class I antigen. Two previous investigations showed an aberrant expression and total loss of HLA class I molecules in human pancreatic cancer (16, 17). The present investigation included substantially more cases into the study, investigated the expression of HLA class I, and compared primary pancreatic carcinoma with tumor metastases.

The present study showed an alteration of HLA class I expression in 61% of primary pancreatic cancer and in 93% of tumor metastases. We believe that these alterations of HLA class I are the possible reason for the non-cytotoxic state of tumor-infiltrating lymphocytes or for the loss of lymphocyte infiltration in some HLA class I–positive tumors. A total loss of HLA class I occurs significantly more frequently in tumor metastases than

(22 months) was considerably higher that of the group with low lymphocyte density (10 months).

Fig. 4 Maximal lymphocyte density of CD8+ cells of HLA class I–negative, heterogeneous, and positive pancreatic tumors. HLA class I–negative tumors showed a low infiltration by T cells. The highest lymphocyte density did not exceed 200 cells/mm². Pancreatic cancer with positive or heterogeneous expression of HLA class I showed a significantly higher maximal lymphocyte infiltration by CD8+ T-cells than HLA class I–negative tumors (P<0.001; Kruskal-Wallis test). Individual values exceeded the density of 200 cells/mm² in most cases.

Fig. 5 Kaplan-Meier analysis of survival time after R0 tumor resection depending on the expression of HLA class I by tumor cells. Statistical analysis showed no significant difference in survival time after R0 resection (A) between negative (median survival: 10.0 months, 95% CI: 6-14 months) and positive/heterogeneous expression of HLA class I (median survival: 11 months, 95% CI: 1-22) (P = 0.75; log-rank test); (B) between low (median survival: 11.0 months, 95% CI: 6-15 months) and high (median survival: 10.0 months, 95% CI: 8-12 months) mean lymphocyte density (P = 0.72; log-rank test); and (C) between low (median survival: 10.0 months, 95% CI: 7-13 months) and high (median survival: 22.0 months, 95% CI: 6-38 months) maximal lymphocyte density (P = 0.15; log-rank test). (Six patients who were alive at last follow-up are censored.)
in primary carcinoma and seems to be present more likely in poorly differentiated rather than in well differentiated or moderately differentiated tumors. This observation indicates that the loss of immunogenic properties can accompany the loss of other phenotypic features such as differentiation. It corresponds well with previous studies which showed identical results in the patients with head and neck squamous cell (18, 19) and prostate cancer (20).

Interestingly, immunohistochemical staining of nonmalignant pancreatic tissue showed a normal positive expression of HLA class I in all pancreatic cell types, except exocrine cells, which represent the major mass of pancreatic tissue. This absence of HLA class I expression was already described in previous studies (21), but the relevance of this observation was not recognized. This finding may have an important implication for the immunology of pancreatic disease owing to the lack of HLA class I pointing towards the pancreas being a site of immunologic privilege.

Using an immunohistochemical analysis, we investigated the cellular immune response and the expression of HLA as a guide for CTLs in human pancreatic cancer. In the present study, we showed that tumor cells of pancreatic carcinoma frequently induce a distinct immune response which results in the infiltration of tumor tissue by T cells. The study of von Bernstorff et al. (22) showed evidence for a state of local and systemic immunosuppression in pancreatic cancer patients. He suggested that the cytotoxic lymphocytes that recognized pancreatic tumor cells do not reach tumor cells sufficiently because they become trapped in the peritumoral tissue. In accordance to this and other previous studies (23, 24), we also found the highest T-cell infiltration in peritumoral tissue. However, we frequently found a high lymphocyte accumulation in pancreatic cancer tissue itself, which has an uneven distribution and should be distinguished into two parts dependent on HLA class I expression on tumor cells. Lymphocytes can diffusely infiltrate the tumor tissue or accumulate locally at high density. The functional relevance of these two types of lymphocyte infiltration seems to be different because of the sites of high lymphocyte accumulation being found only in tumors exhibiting a positive/heterogeneous expression of HLA class I. Thus, the recognition of human pancreatic cancer by cytotoxic lymphocytes was only possible if the molecule complex of HLA class I was positively expressed by at least one part of the tumor cells. In contrast to HLA class I–positive tumors, the total loss of HLA class I by pancreatic tumor cells uniformly led to an absent infiltration by cytotoxic T-cells. We believe that these findings are crucial for the clinical immunotherapy of pancreatic cancer because the patient selection with HLA class I–expressing tumors is necessary for an effective T-cell–based immunotherapy.

The HLA class I complex has potential prognostic relevance for cancer patients due to its leading role in the immune recognition of antigenic cells. However, previous studies provide contradictory results about the impact of HLA class I on the survival. Several studies showed that the down-regulation of HLA class I correlated with better patient survival in colorectal cancer (25) and uveal melanoma (26). These studies postulated the important role of natural killer cells in the elimination of HLA class I–negative cells. Other investigations showed a better outcome of reduced HLA class I expression in breast (27) and prostate cancer (20). One study reported about the missing correlation between HLA class I and survival in cutaneous melanoma (28). In the present study, the median survival was not significantly different in patients with positive or heterogeneous/negative expression of HLA class I. Immunohistochemistry is the most useful method for the qualitative analysis of antigen expression by single tumor cells on histologic sections. However, this technique does not allow the measurement of exact antigen concentration, which could be a better factor for patient survival.

The majority of lymphocytes infiltrating pancreatic tumors express CD8 and CD45R0 cell surface molecules and therefore represent a mature and potentially cytotoxic stage. These findings correspond well to previous reports investigating CD8 and CD45R0 markers in pancreatic carcinoma (23, 24). The crucial aspect of lymphocyte infiltration is whether the immune response against the tumor is restricted to tumor recognition and migration into tumor tissue or whether tumor-infiltrating lymphocytes destroy tumor cells. Some previous clinical studies of ovarian (29), colorectal (30, 31), gallbladder (32), esophageal (33), breast (34), lung (35) cancer, and melanoma (36) showed that infiltrating lymphocytes can exploit their tumor-cytotoxic potential owing to the patients with high lymphocyte infiltration showing a significantly better prognosis than the patients with low lymphocyte infiltration. The present study showed a higher median survival time of patients with high infiltration (22 months) than with low infiltration (10 months) by cytotoxic cells after R0 resection.

In summary, we investigated the role of HLA class I expression in the T-cell–mediated immune response in human pancreatic carcinoma. This investigation showed that pancreatic cancer induced a cellular immune response which resulted in an infiltration of tumor tissue by specific T-lymphocytes. Cytotoxic T-cells in part recognize malignant cells and migrate into the tumor if the tumor cells express HLA class I molecules. High infiltration by cytotoxic cells showed a trend towards an improved median survival time after surgical resection.

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