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Natural Killer cells infiltrating colorectal cancer and MHC class I expression

RUNNING TITLE: Natural Killer cells in colorectal cancer

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ABSTRACT

A majority of colorectal adenocarcinomas displays diminished MHC class I expression, making them particularly vulnerable for NK cell-mediated killing. Generally, these tumors also show a substantial inflammatory infiltrate. Most inflammatory cells, however, reside in the tumor stroma, where they do not have direct contact with tumor cells in the tumor epithelium. In this study we investigated the correlation between colorectal tumor MHC class I aberrations and infiltration of NK cells. We studied 88 tumor specimens obtained from 88 colorectal cancer patients for locus specific HLA aberrations and correlated these data to infiltration of CD4, CD8 and CD56 positive lymphocytes. The lymphocyte markers were individually combined with laminin as a second marker to facilitate quantification in the different tumor compartments, i.e. tumor epithelium and tumor stroma. Locus specific partial -or total HLA class I loss was detected in 72% of the tumors studied. Twenty-eight percent had no HLA loss at all. Mean overall intra-epithelial infiltration of CD56 positive lymphocytes was 7 cells per mm² compared to 76 cells per mm² for CD8 and 19 cells per mm² for CD4 positive lymphocytes. Locus specific partial or total loss of tumor cell MHC class I expression was positively correlated with the intra-epithelial infiltration of CD8 positive cells ($p = 0.01$), but not with CD4 or CD56 positive lymphocytes. Triple immunofluorescence staining showed that these cells were CD8 -and granzyme-B positive T-lymphocytes. Our data showed that colorectal tumors are sparsely infiltrated by CD56 positive cells compared to CD8 positive T-cells and that loss of MHC is associated with T cell infiltration instead of NK cell infiltration. Considering the fact that MHC loss is quite common in colorectal cancer and that, due to local absence of NK cells, it is unlikely that there has been selection for NK-escape variants, improvement of the intra-epithelial infiltration/migration of NK cells may be an important basis for the development of an effective adjuvant NK-based immunotherapy of colorectal cancer.

INTRODUCTION

Colorectal cancer is the most common gastrointestinal cancer in the world and potentially curable with surgical resection of the primary tumor. The clinical problem of colorectal cancer, however, is the spread and outgrowth of metastases. Once the disease has spread to distant organs, treatment options are limited to aggressive systemic therapies with high treatment-related morbidity and/or mortality, while a cure can rarely be obtained? Development of new adjuvant therapeutic strategies focuses on more selective and safer therapeutic options, including immunotherapy. The major advantage of using immune-based adjuvant therapeutic strategies is the potentially high selective focus of immune effector cells on malignant cells, which may limit treatment-related morbidity and/or mortality. However, there are still questions unanswered concerning the immunological mechanisms in an anti-tumor response. A classical way to gain insight in the immunological mechanisms in the host defense against malignant cells, is to evaluate the histopathology of the host' natural infiltration patterns¹.

The leukocyte infiltrate of primary colorectal tumors is presumed to represent the natural defensive activity of the host against the tumor. A majority of infiltrating leukocytes consists of T-lymphocytes infiltrating the stromal compartment of the tumor. These infiltrating immune cells do not seem to interact directly with the tumor cells, since they are separated by stromal structures (i.e. basal membrane-like structures) that seemingly form a physical barrier preventing interaction². Colorectal tumors show a varying amount of leukocytes in direct contact with tumor cells, i.e. in the tumor-epithelial compartment. This leukocyte fraction consists mainly of CD8 positive lymphocytes³. It is generally presumed that these cells are specific anti-tumor CTL. However, as different types of leukocytes may express CD8, among which NK cells, the exact background of these cells still remains unclear.

In vitro, NK cells function well as effector cells against tumor target cells⁴⁻⁶. In vivo however, NK cells migrating from the bloodstream into a solid tumor only form a minor fraction of the total tumor-infiltrating leukocyte population⁷. Their exact loco-regional function in relation to the tumor is still obscure. One major significance of NK cells is believed to lay in the clearance of tumor cells lacking classical MHC class I surface molecules. Several studies have shown that 70-90% of colorectal tumors show aberrant MHC class I expression⁸⁻¹¹.

To investigate the possible relationship between loss of MHC class I expression and NK cell infiltration in colorectal cancer, we evaluated a series of 88 colorectal tumors obtained from 88 patients for their MHC class I expression and type of leukocyte infiltration using immunohistochemistry and immunofluorescence.

MATERIALS AND METHODS

Patients

A randomly selected group of 88 colorectal cancer patients from a previously described database of consecutive colorectal cancer patients was analyzed¹². Clinical -and histopathological data are shown in Table 1. Tumors were evaluated for differentiation grade and lymphocytic infiltration according to Jass’ criteria¹³ on hematoxylin-eosin stainings. Patient follow-up was completed until January 2003.

Table 1

	<i>n</i>
Gender	
Female	36
Male	52
Age	
0–50	12
>50	76
Location ^a	
Right-sided	33
Left-sided	55
Tumor stage	
Stage II	38
Stage III	50
Differentiation	
Poor	54
Moderate	20
Well	11
Unassessable	3
Recurrences	
No	58
Yes	30

Patient and tumor characteristics of 88 curatively resected colorectal cancer patients. Parameters were assessed according to standard clinical and pathology protocols.
*Right-sided location: caecum – flexura lienalis

Immunohistochemistry

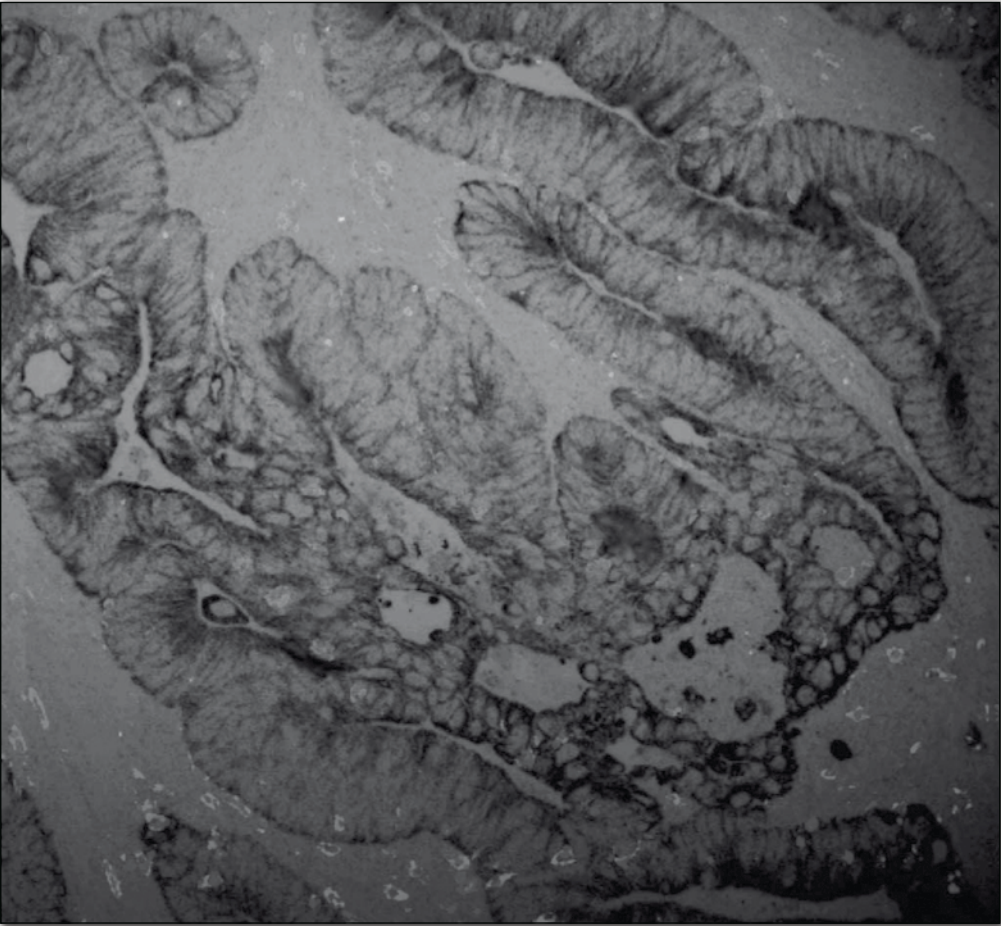
Tissue sections were stained as described by Menon et al^{14,15}. Either one of the following monoclonal antibodies (culture supernatant) for immunohistochemical staining was used: mouse anti-human HLA-A (Clone HCA2, isotype IgG1, generously provided by dr. J. Neefjes, NKI, Amsterdam), mouse anti-human HLA-B/C (Clone HC10, isotype

IgG2a, generously provided by dr. J. Neefjes, NKI, Amsterdam)¹⁶, mouse anti-human CD4 (Clone 1F6, isotype IgG1, Novocastra Ltd, Newcastle, UK), mouse anti-human CD8 (Clone 4B11, isotype IgG2b, Novocastra Ltd, Newcastle, UK) and mouse anti-human CD56 (Clone 123C3, isotype IgG1, Zymed Inc, San Francisco, USA). Four micrometers thick paraffin sections were mounted on aminopropylethoxysilane (APES) coated slides, and dried overnight at 37°C. Tissue sections were de-paraffinized and rehydrated. Endogenous peroxidase was blocked for 20 minutes in 0.3% hydrogen-peroxide methanol. Antigen retrieval was achieved by boiling in 10mM citrate buffer (pH = 6.0) for 10 minutes in a microwave oven. After washing in PBS the slides were incubated overnight at room temperature with primary antibodies. Sections for HCA2 and HC10 were washed in PBS and incubated with biotinylated rabbit-anti-mouse (1:200, DAKO, Glostrup, Denmark) for 30 minutes, washed again with PBS, and incubated with Streptavidin-Biotin-Complex (DAKO, Glostrup, Denmark) for 30 minutes. Sections for other antibodies were washed and incubated with mouse Envision labeled with Horse Radish Peroxidase (m-Envision^{HRP}) for 30 minutes, washed in PBS and rinsed in 0.05M Tris-HCl buffer (pH 7.6) for 5 minutes. Sections were washed and developed in 3,3-di-amino-benzidine (DAB) tetrahydrochloride substrate solution containing 0.002% hydrogen-peroxid, for 10 minutes, resulting in a brown staining. HCA2 and HC10 stained sections were counterstained with hematoxylin and mounted with pertex (Histolab, Göteborg, Sweden). Lymphocyte stained sections were subsequently incubated in 0.01% trypsin in 0.1 mM CaCl₂ for 10 minutes. After washing in demineralized water, sections were incubated overnight with a rabbit polyclonal antibody against laminin (Sigma-Aldrich, Zwijndrecht, The Netherlands). After washing, sections were incubated with a swine-anti-rabbit conjugate labeled with biotin (DAKO) for 30 minutes. Subsequently, sections were incubated for 30 minutes with Streptavidin-Biotin-complex (DAKO) labeled with alkaline phosphatase. Sections were developed in a NBT/BCIP solution, resulting in a blue signal.

For additional immunofluorescent staining we selected 6 tumors with absent HLA-A and B/C expression. Sections were initially treated as described above except that the endogenous peroxidase blocking step was replaced for a 20 minute incubation with 10% human AB serum followed by a 10 minute incubation with 0.1% cationic BSA (Aurion, Wageningen, The Netherlands). Antigen retrieval was performed by boiling in 1 mM EDTA for 10 minutes. First overnight antibody incubation was done with an antibody directed against cytokeratin 8 (DAKO). Next we incubated for 2 hours with ultra-small gold (USG) labelled Goat-anti-Mouse (Aurion) diluted in

0.1% w/v BSAc in PBS. After washing three times with MQ a 40 min-ute silver enhancement was performed using a silver enhancement kit (Aurion). After washing, sections were consecutively incubated overnight with a mixture of two monoclonal antibodies: mouse anti-human CD56 (Clone 123C3, Zymed Inc, San Francisco, USA), mouse anti-human Granzyme-B (Clone GRB-7, Monosan, Uden, The Netherlands) and one rabbit polyclonal: anti-CD3 (rabbit polyclonal, Abcam). Next slides were incubated with a mix of three matching fluorescent conjugates: goat-anti-rabbit-IgG2a-Alexa-546, goat-anti-mouse-IgG1-Alexa-488 and goat-anti-mouse-IgG2a-Alexa-647 (Molecular Probes Inc, Leiden, The Netherlands), for 1 hour followed by washing in PBS. Slides were mounted in Mowiol mounting me-dium and stored in the dark at 4°C until scanning.

Figure 1



Tumor-infiltrating lymphocytes in a HLA-I downregulated colorectal tumor. Combined silver-gold immunofluorescence staining of a HLA-I down-regulated colorectal tumor. Picture shows both stromal and intra-epithelial infiltrating CD3 (red) and granzyme B (blue) positive lympho-cytes. Only few CD56 positive cells (green) are present in the tumor stroma. Bright-field microscopy reveals the cytokeratin labeled (dark-grey) tumor epithelial fields.

Microscopic evaluation of tumor sections

Pictures of the immunofluorescent slides were taken with a confocal Laser Scanning Microscope (Zeiss LSM510; Zeiss, Jena, Germany) in a multi-track setting. For the detection of the silver stain we used bright field microscopy using the 633nm laser in a very low intensity to prevent excitation of the Alexa-647 fluorochrome. Microscopic analysis was performed separately by two observers who had no knowledge of the clinical outcome of the patients. The percentage of the tumor cells expressing HLA-A and HLA-B/C was estimated in each case. Eventually in all cases a consensus was met. Normal HLA expression was defined as a situation in which all tumor cells expressed HLA. HLA expression was defined as reduced when tumor cells showed partial absence of either HLA-A or B/C. Total loss of HLA expression was noted when no tumor cell expressed HLA-A or B/C. The tumor stroma (fibroblasts, lymphocytes, endothelial cells) served as an internal positive control.

Table 2

Intra-epithelial lymphocytes	HLA class I expression					P-value
	Normal (mean cells per mm ²)	n	Reduced (mean cells per mm ²)	n	Absent (mean cells per mm ²)	
CD4	8	21	9	56	27	2 0.13
CD8	18	19	79	56	203	2 0.01
CD56	5	21	8	54	6	2 0.30

The number of intra-epithelial lymphocytes according to the HLA-I status of the tumor cells. Nine tumor specimens were not evaluated for infiltration by CD4+ cells and 11 cases for both CD8+ and CD56+ cells.

Tumor-infiltrating lymphocytes according to HLA class I aberrations
The number of intra-epithelial lymphocytes according to the HLA-I status of the tumor cells. Nine tumor specimens were not evaluated for infiltration by CD4-positive cells and 11 cases for both CD8- and CD56-positive cells.

Statistical analysis

All statistical analyses were done using the SPSS software package (SPSS, Chicago, Illinois, USA). Disease-free survival data were ana-lyzed using Kaplan-Meier survival estimation and the log-rank test was used for comparison of the survival curves. Statistical analy-ses between groups were performed using the Chi-squared test for comparing proportions and Krukas Wallis test for comparing means. P values less than 0.05 were considered significant.

RESULTS

Patient characteristics

A panel of 88 primary tumors of colorectal origin was investigated. The patients’ characteristics and clinicopathological parameters are

given in Table 1. The panel consisted of about equal numbers of stage II (Dukes B; n=38, 43%) and stage III (Dukes C; n=50, 57%) tumors. The average age of the patients was 66.9 years (range: 26.0 – 85.0 years). As expected, tumor stage significantly correlated inversely with the time of disease-free survival (p=0.002, data not shown). None of the other patient -or tumor characteristics as described in Table 1 correlated with DFS.

HLA-I expression

Locus specific down-regulation was detected in 63 (72 %) of 87 colorectal tumors, using antibodies against HLA-A and HLA-B/C (resp. HCA2 and HC10). One case could not be evaluated due to the absence of HLA-A signal on repeated evaluations. In 6 (7 %) tumors HLA expression was absent, i.e. there was no signal detectable for either HLA-A or B/C. Twenty-four tumors (28 %) had no HLA loss at all. HLA-A expression correlated significantly with a longer disease-free survival (p = 0.02).

Tumor-infiltrating lymphocytes

Mean overall intra-epithelial infiltration of CD56 positive lymphocytes was 7 cells per mm² versus 76 cells per mm² for CD8 and 10 cells per mm² for CD4 positive lymphocytes. Immuno-histochemical analysis of primary tumor sections revealed that locus specific MHC aberrations significantly correlated with the intra-epithelial infiltration of CD8 positive cells (p = 0.02), but not with CD4 or CD56 positive lymphocytes (Table 2). We performed an additional immunofluorescent triple staining on the total HLA-I down-regulated tumors and found that the majority of the intra-epithelial infiltrating cells consisted of CD3 -and Granzyme-B positive lymphocytes (Figure 1). These results show that not NK cells, but T cells preferentially infiltrate colorectal tumor specimens that show down-regulated MHC class I expression.

DISCUSSION

Ample studies have shown an accumulation of inflammatory cells in the direct vicinity of solid tumors¹⁷⁻²⁰. In most studies, patients with a relatively dense infiltrate performed better in the clinical outcome. Detailed analysis of this infiltrate in various adenocarcinomas showed that only cells infiltrating the tumor-epithelium, i.e. cells in direct contact with tumor cells, contributed to this survival benefit²¹⁻²³. NK

cell infiltration in colorectal cancer has not been studied extensively in the past. Several studies found that the infiltration of NK cells in malignant tumors was associated with a favorable outcome²⁴⁻²⁶. However, they used an antibody against CD57 to identify NK cells, which is not an exclusive NK cell marker, since it is also expressed on a subset of T-lymphocytes. We recently found that the majority of infiltrating CD57 positive cells in colorectal tumors also expressed the T-cell receptor (data not shown). We therefore used the marker CD56, which however is not an exclusive NK cell marker, since it is also expressed on a subset of T lymphocytes, but in combination with CD3 we were able to distinguish these subpopulations.

Other studies have shown that loss of MHC class I expression is quite common in colorectal cancer and that these patients show a survival benefit^{8,9,27-31}. We asked ourselves whether this survival benefit could be due to specific infiltration in tumors showing loss of MHC class I expression. We found that both CTL as well as NK cells infiltrate colorectal tumors, but the NK cell fraction is relatively small, especially in the tumor-epithelium. Furthermore, our staining demonstrated that tumors showing loss of MHC class I expression were more vigorously infiltrated by CD8 positive lymphocytes. At first we hypothesized that these were CD8 positive NK cells, but our triple immunofluorescence staining surprisingly revealed that the majority of these intra-epithelial infiltrating CD8 cells also carried the T-cell receptor CD3 and did not express CD56. It remains unclear why HLA-I aberrant tumors contain significantly more intra-epithelial CD8 positive T-lymphocytes than HLA intact tumors. Further characterization of these cells, as to their exact T cell receptor ($\alpha\beta$ or $\gamma\delta$), is necessary to illuminate their role and function. It is possible that in these tumors, due to T cell-mediated tumor cell killing, there have been a selection for MHC class I negative variants and that these tumors have maintained the capacity to attract T cells.

In a previous study we found that patients with primary tumors showing loss of MHC class I expression developed fewer distant metastases. Therefore, it is possible that the survival benefit is due to the fact that metastasizing tumor cells in these patients are efficiently cleared by NK cells in the circulation. This hypothesis is further supported by the observation that in a paired series of primary colorectal tumor and distant metastases from the same patient, less loss of MHC class I was observed than in a random series of primary colorectal cancer³². These data suggest that NK cells play an important role in the prevention of metastatic spread rather than locally in the primary tumor.

We hypothesize that if NK cells are capable of infiltrating solid tumors, they may kill tumor cells that show loss of MHC class I expression, and thus may contribute to treatment of colorectal cancer. The success of such NK based immunotherapy will depend on the ability of NK cells to infiltrate the tumor-epithelium. If this can be attained, for example through activation of NK cells by specific pro-infiltration chemokines/ cytokines, NK cells potentially form an effective immunotherapeutic basis.

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