The Prognostic Significance of Intratumoral Natural Killer Cells in Patients with Colorectal Carcinoma

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BACKGROUND. Natural killer (NK) cells have a spontaneous cytotoxic capacity against tumor cells. These cells represent a small proportion of human colon carcinoma-infiltrating lymphocytes. Their prognostic significance in these tumors has yet to be determined.

METHODS. One hundred and fifty-seven patients who each had a colectomy for large bowel adenocarcinoma were studied. No patient received adjuvant therapy. Immunohistochemical stains were performed for NK cells using the monoclonal antibody CD57. The number of NK cells was counted using a MICRON image analyzer. The total area studied for each tumor was 1 cm². In this area, 50 intratumoral fields of 0.173 mm² were selected. The degree of NK infiltration was classified as little (<50 NK cells), moderate (50–150 NK cells), and extensive (>150 NK cells). The Kaplan-Meier method was used to obtain survival figures. Multivariate analyses were performed using the Cox regression model.

RESULTS. At 5 years, patients with little and moderate NK infiltration showed significantly shorter survival rates (overall and disease free survival) than those with extensive infiltration (P < 0.01). Three significant factors affecting survival were selected in a stepwise fashion in increasing order as follows: TNM stage, NK infiltration, and lymphocytic infiltration. Patients with TNM Stage III disease and extensive NK infiltration showed significantly longer survival rates than those with little or moderate infiltration (P < 0.001). In these patients, multivariate analysis using the Cox regression model identified two significant variables: number of involved lymph nodes and NK cell infiltration.

CONCLUSIONS. In patients with colorectal carcinoma, an extensive intratumoral infiltration of NK cells is associated with a favorable tumor outcome. Intratumoral infiltration of NK cells can be used as a variable with prognostic value, especially in patients with TNM Stage III disease. Cancer 1997;79:2320–8.

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KEYWORDS: colorectal carcinoma, prognostic factor, natural killer cell, multivariate analysis, survival rate.

It is very important for clinicians to know the expected outcome of a patient with colorectal carcinoma to plan appropriate therapy. Traditionally, the depth of penetration of the tumor through the bowel wall combined with the presence or absence of lymph node and distant metastases have been considered the best prognostic parameters in colorectal carcinoma. In 1932, Dukes1 published a pathologic staging system based on the spread of rectal carcinoma and, with some modifications,2,3 it is still considered the best prognostic indicator of survival in patients with colorectal carcinomas. However, due to the lack of prognostic precision offered by the Dukes staging system,1 other prognostic variables also have been investigated to obtain prognostic information.4 Among them, special attention has been di-
FIGURE 1. Immunoperoxidase staining of colorectal tumors with the monoclonal antibody IOT-10 (CD57). (A) Rectal carcinoma with "little" intratumoral infiltration of natural killer (NK) cells. (B) Carcinoma of the colon with "moderate" intratumoral infiltration of NK cells. (C) Carcinoma of the colon with "extensive" intratumoral infiltration of NK cells. This section shows an abundant NK cell infiltration and a Crohn's-like lymphoid reaction in the advancing edge of tumor (original magnification ×200). (D) Photomicrograph of a tumor with "extensive" infiltration of NK cells showing two NK cells in contact with tumoral cells. Arrows indicate selected specifically staining cells (original magnification ×400).
FIGURE 2. Kaplan–Meier survival curves by pathologic features: (a) Survival by TNM stage. (b) Survival by natural killer (NK) cell infiltration. (c) Survival by NK cell infiltration. Patients with little and moderate NK cell infiltration are grouped in Figures 2e, 2f, and 2g. Kaplan–Meier survival curves by NK cell infiltration are shown according to TNM stage.

rected to the inflammatory infiltrates as a reaction reflecting host resistance against malignancy.5–8 Furthermore, lymphocytic infiltration has been considered to be a good prognostic factor in colorectal carcinomas5–13 and some authors have suggested that a subset of these lymphocytes with a spontaneous cytotoxic capacity against tumor cells, the natural killer (NK) cells, can play an important role in the prognosis of intestinal tumors.14,15 These cells have a particular role in immunosurveillance against neoplasms and represent a small proportion of human colon carcinoma-infiltrating lymphocytes.16

The purpose of the current study was to evaluate the prognostic potential of intratumoral NK cell infil-
TABLE 1
Influence of Tumoral Stage on 5-Year Survival

| Stage | No. of patients | 5-year cumulative survival rate (%) | P value by log rank test |
|-------|----------------|--------------------------------------|--------------------------|
| I     | 44             | 88.6                                 | P < 0.001                |
| II    | 58             | 78.7                                 |                          |
| III   | 55             | 25.4                                 |                          |

Eleven patients with insufficient biopsy specimens for a correct evaluation of all investigated parameters were excluded. The paraffin embedded sections were stained with hematoxylin and eosin. Immunohistochemical stains were performed for NK cells using the monoclonal antibody IOT-10 (CD57) (1:50 diluted; Immunotech SA, Marseilles, France) incubated overnight at 4°C. The monoclonal antichromogranin antibody (prediluted; Biogenex, San Ramon, CA) was used to stain neuroendocrine cells. Primary antibodies were visualized with a streptavidin-biotin-peroxidase supersensitive kit (Biogenex).

The tumors were staged using the TNM classification described by Hutter and Sobin, which closely corresponds to Dukes staging in four main categories (Stage I: tumor invades into the submucosa or the muscularis propria, with no lymph node metastasis; Stage II: tumors extend through the muscularis propria without lymph node metastasis; and Stage III: tumors with lymph node metastasis). Patients with distant metastases (Stage IV) were excluded.

The number of NK cells was counted with a Nikon binocular microscope using a MICRON (MICRON Spain S.A., Barcelona, Spain) image analyzer with the MIP-interactive method. The total area studied for each tumor was 1 cm². In this area, the authors selected 50 intratumoral fields at a magnification of ×200 (area of field: 0.173 mm²) (in the border of the tumoral invasion the authors considered only a microscopic high-power field around the neoplastic glands). Tissue sections were analyzed by two independent observers. Degree of NK infiltration was classified as little (if 50 NK cells could not be found in 50 intratumoral fields), moderate (if >50 and <150 NK cells were found in 50 intratumoral fields), and extensive (when >150 NK cells could be found in <50 intratumoral fields) (Figs. 1a, 1b, and 1c). Seven cases with no interobserver agreement were excluded, leaving 157 cases to be included in this study.

TABLE 2
Influence of Natural Killer Cell Infiltration on 5-Year Survival

| NK cell infiltration | No. of patients | 5-year cumulative survival rate (%) | P value by log rank test |
|----------------------|----------------|--------------------------------------|--------------------------|
| Little               | 61             | 50.8                                 | P = 0.0032               |
| Moderate             | 71             | 56.3                                 | P = 0.388*               |
| Extensive            | 25             | 92                                   | P = 0.0031*              |

NK: natural killer.

* P value of survival differences between patients with little vs. moderate infiltration of natural killer cells.

† P value of survival differences between patients with moderate vs. extensive infiltration of natural killer cells.

TABLE 3
Influence of Natural Killer Cell (NK) Infiltration on 5-year Survival. Patients with Little or Moderate Infiltration of Cells are Grouped

| NK cell infiltration | No. of patients | 5-year cumulative survival rate (%) | P value by log rank test |
|----------------------|----------------|--------------------------------------|--------------------------|
| Little/moderate      | 132            | 53.7                                 | P = 0.0011               |
| Extensive            | 25             | 92                                   |                          |

NK: natural killer.

Histo logic Specimens
Histologic analysis was performed in 175 patients. Eleven patients with insufficient biopsy specimens for a correct evaluation of all investigated parameters were excluded. The paraffin embedded sections were stained with hematoxylin and eosin. Immunohistochemical stains were performed for NK cells using the monoclonal antibody IOT-10 (CD57) (1:50 diluted; Immunotech SA, Marseilles, France) incubated overnight at 4°C. The monoclonal antichromogranin antibody (prediluted; Biogenex, San Ramon, CA) was used to stain neuroendocrine cells. Primary antibodies were visualized with a streptavidin-biotin-peroxidase supersensitive kit (Biogenex).

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Other Histologic Variables Studied

Pattern of growth
Tumors were defined as expanding or infiltrating following the morphologic criteria suggested by Jass et al. Expanding tumors were those with a circumscribed or pushing border of growth whereas infiltrating tumors invaded in a diffuse fashion with an ill-defined margin. There were 78 expanding tumors and 79 infiltrating tumors.

Degree of differentiation
Adenocarcinoma, well-differentiated indicated tumors comprised entirely of glandular formation. Adenocarci-
TABLE 4
Natural Killer Cell Infiltration and Survival Rates in Relation to Tumoral Stage

| Stage | NK cell infiltration | No. of patients | 5-year cumulative survival rate (%) | P value by log rank test |
|-------|----------------------|-----------------|------------------------------------|-------------------------|
| I     | Little/mod          | 35              | 85                                 | P = 0.24                |
|       | Extensive           | 9               | 100                                |                         |
| II    | Little/mod          | 52              | 67.3                               | P = 0.11                |
|       | Extensive           | 6               | 100                                |                         |
| III   | Little/mod          | 45              | 13.3                               | P = 0.0008*             |
|       | Extensive           | 10              | 80                                 |                         |

NK: natural killer; mod: moderate.
*P value of survival differences between Stage III patients with little/moderate vs. extensive infiltration of natural killer cells.

noma, moderately differentiated indicated tumors comprised of solid sheets of malignant cells admixed with glandular formations. The solid sheets comprised at least 25% of the tumor. Adenocarcinoma, poorly differentiated indicated tumors with <25% of the neoplastic cells forming glands. There were 105 well-differentiated tumors, 45 moderately differentiated tumors, and 7 poorly-differentiated tumors.

**Mucinous pattern**
A tumor was considered mucinous when a mucinous component was present in >50% of the tumor section (16 cases).

**Peritumoral lymphocytic infiltration**
The authors followed the criteria used by Jass et al. Extensive infiltration (62 cases) indicated the presence of an inflammatory band at the advancing tumor margin in which lymphocytes were the most abundant cellular component. Absence or extreme paucity of lymphocytes (11 cases) was recorded as little infiltration. An intermediate pattern of infiltration was defined as moderate infiltration (84 cases).

**Number of involved lymph nodes**
N1 indicated 1–3 metastatic lymph node (31 cases). N2 indicated ≥4 metastatic lymph node (24 cases).

**Follow-Up**
Patients were followed for at least 5 years or until death. Both overall survival and relapse free survival were examined. The overall survival was measured from the time of resection of the tumor until death. Eight patients (5%) were lost to follow-up, 1 at 24 months, 1 at 38 months, and 6 between 41 and 50 months. At 5 years, 63 patients (40.1%) had died, all with recurrent disease. Seventy patients developed recurrence during follow-up: 48 distant metastases and 22 local or regional recurrences.

The main sources of information on clinical outcome were the clinical and pathology reports and in a few cases by direct telephone interviews with close relatives of the patients.

**Statistical Analysis**
The Kaplan–Meier method was used to obtain cancer survival figures. Differences in observed survival between groups were tested for statistical significance using the log rank test. Multivariate analysis was performed by the Cox stepwise regression model. Statistical analysis was performed using the SPSS for Windows 6.1 program (SPSS Inc., Chicago, IL).

**RESULTS**

**Overall Survival**
Of the 157 tumors staged by TNM classification, 44 were Stage I, 58 were Stage II, and 55 were Stage III. The influence of stage on survival is shown by Kaplan–Meier curves (Fig. 2a) and Table 1 shows the corresponding data. As expected, prognosis was significantly related to staging (P < 0.001).

In the study of NK cell intratumoral infiltration, a significant difference in survival was found between groups of patients with little or moderate versus those with extensive NK cell infiltration (P = 0.0032) (Table 2) (Fig. 2b). However, there was no significant difference between patients with little versus those with moderate NK cell infiltration (P = 0.388) (Table 2).

The statistical differences were significant (P = 0.0011) when the authors compared the cumulative survival curves of patients with little or moderate versus those with extensive NK cell infiltration (Table 3) (Fig. 2c).

The authors also studied the influence of NK cell intratumoral infiltration on the survival of patients with TNM Stage I, II, and III disease. Survival rates are summarized in Table 4 and shown in Figures 2d, 2e, and 2f. The survival difference between patients with...
FIGURE 3. Kaplan–Meier disease free survival curves by a) TNM tumoral stage, b) natural killer (NK) cell infiltration, and c) NK cell infiltration. Patients with little and moderate NK cell infiltration are grouped in Figures 3e, 3f, and 3g. Kaplan–Meier disease free survival curves are shown by NK cell infiltration according to TNM stage.

little or moderate versus those with extensive NK cell infiltration was not statistically significant in patients with TNM Stage I and II disease ($P = 0.24$ and $P = 0.11$, respectively) but was very significant in patients with TNM Stage III disease ($P = 0.0008$).

Disease Free Survival

**Disease free survival in relation to TNM stage**

The 5-year relapse free survival rate of patients with Stage I tumors ($n = 44$) was 89%, was 64% for those with Stage II tumors ($n = 58$), and was 24% for those
TABLE 5
Analysis by the Cox Regression Model

| Step | Variable added | P value | Close alternatives |
|------|----------------|---------|--------------------|
| 1    | TNM stage      | < 0.001 |                     |
| 2    | NK             | < 0.001 | Lymphocytes (P < 0.001) |
| 3    | Lymphocytes    | 0.012   |                     |

Disease free survival: variables in the equation

| Step | Variable added | P value | Close alternatives |
|------|----------------|---------|--------------------|
| 1    | TNM stage      | < 0.001 | Lymphocytes (P < 0.001) |
| 2    | NK             | 0.002   |                     |
| 3    | Lymphocytes    | 0.018   |                     |

NK: natural killer.
Other variables considered: differentiation, pattern of growth, age > 65 years, mucinous pattern, and gender.

TABLE 6
Analysis by the Cox Regression Model in Patients with TNM Stage III Disease

| Step | Variable added | P value | Close alternatives |
|------|----------------|---------|--------------------|
| 1    | Lymph nodes    | < 0.001 | Lymphocytes (P = 0.001) |
| 2    | NK             | < 0.001 | Lymphocytes (P = 0.008) |

Disease free survival: variables in the equation

| Step | Variable added | P value | Close alternatives |
|------|----------------|---------|--------------------|
| 1    | Lymph nodes    | 0.001   | Lymphocytes (P = 0.006) |
| 2    | NK             | 0.002   | Lymphocytes (P = 0.01) |

NK: natural killer.
Other variables considered: differentiation, pattern of growth, age > 65 years, mucinous pattern, and gender.

with Stage III tumors (n = 55). The difference in the disease free survival rate was statistically significant (P < 0.001). (Fig. 3a).

Disease free survival in relation to NK cell infiltration
The 5-year relapse free survival rate of patients with extensive NK cell infiltration (n = 25) was 80%, was 52% for those with moderate NK cell infiltration (n = 71), and was 49% for those with little NK cell infiltration (n = 61). The difference in the disease free survival rate dependent of NK cell infiltration was statistically significant (one-sided log rank test; P = 0.0239) (Fig. 3b). No statistically significant difference was found between patients with little versus those with moderate NK cell infiltration (P = 0.5071). Patients with little/moderate NK cell infiltration (n = 132) had a 5-year disease free survival of 51% and those with extensive NK cell infiltration (n = 25) had a 5-year disease free survival rate of 80%. The difference in the disease free survival rates was statistically significant (P = 0.0084) (Fig. 3c).

Patients with extensive NK cell infiltration and Stage I tumors (n = 9) and Stage II tumors (n = 6) had a 5-year relapse free survival rate of 100%. Patients with Stage I tumors and little or moderate NK cell infiltration (n = 35) had a 5-year disease free survival rate of 86% and those with a Stage II tumor and little/moderate NK cell infiltration (n = 52) had a 5-year disease free survival rate of 60%. The difference in the disease free survival rate was not statistically significant (patients with Stage I tumor: P = 0.2425 and patients with Stage II tumors: P = 0.0731) (Figs. 3d and 3e).

The 5-year relapse free survival rate of patients with Stage III tumors and extensive NK cell infiltration (n = 10) was 50% and was 13% for of patients with Stage III tumors and little/moderate NK cell infiltration (n = 45). The difference in the disease free survival rate was statistically significant (P = 0.0083) (Fig. 3f).

Multivariate Analysis
In the multivariate regression analysis, TNM stage, NK cell infiltration, and peritumoral lymphocytic infiltration were the primary factors influencing both overall survival and disease recurrence (Table 5). Pattern of growth, degree of differentiation, mucinous pattern, age, and gender were not selected in the current multivariate analysis. In patients with Stage III disease, the main prognostic factors were the number of lymph nodes involved by the tumor (> three) and NK cell infiltration; no other variables entered into the equation (Table 6).

DISCUSSION
The main purpose of this investigation was to evaluate in colorectal carcinomas the prognostic significance of NK cells, a type of cell that forms part of tumoral inflammatory infiltrates. Many authors have studied lymphoid infiltrates in colorectal carcinomas, especially at the periphery of invasive tumors. However, because the cytotoxic activity of NK cells is activated by contact with neoplastic cells, in the current study only NK cells located in lymphocytic infiltrates among and around the tumoral glands were examined. The authors chose the monoclonal antibody IOT-
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10 (CD57) for identification of NK cells because they had previously observed that it immunostained these cells consistently and accurately in paraffin embedded specimens.\textsuperscript{21,22} This antibody recognizes a carbohydrate antigen present on a subset of peripheral blood mononuclear cells involved in NK activity. The antigen is also expressed in neuroendocrine cells. In the current study, the majority of CD57 positive cells in the lymphocytic infiltrates were found around the tumoral glands. However, isolated CD57 positive cells occasionally were located very close to tumoral cells or within the epithelium of neoplastic glands, similar to the usual disposition of neuroendocrine cells of the normal colonic epithelium. In these cases, the immunohistochemical stain with chromogranin antibody was always negative both in these cells and in the tumor cells.

With regard to the influence of NK cell intratumoral infiltration on overall survival and disease free survival at 5 years, the differences between survival curves of patients with little or moderate versus those with extensive NK cell infiltration were very significant in the current study. Therefore, extensive NK cell infiltration is a manifestation of a good immunoresponse of the host against the tumor and is associated with a good prognosis.

Nevertheless, the authors wanted to examine whether the survival influence of NK cell intratumoral infiltration could also be related to the tumoral stage. They found that patients with TNM Stage III tumors and extensive NK cell infiltration had significantly longer survival than those with the same tumoral stage and little or moderate NK cell infiltration. However, in spite of the high survival rate of patients with TNM Stage I or II tumors and extensive NK cell infiltration, prognosis was not related to the degree of NK cell infiltration in these patients. The reason for this is probably that a 5-year follow-up is insufficient, especially in patients with a generally high rate of 5-year survival, and it would be necessary to continue the follow-up for a longer period of time in this group of patients.

To assess the relative prognostic value of the NK cell tumoral infiltration, the authors studied other prognostic variables in a multivariate analysis using the Cox stepwise regression model (TNM stage, lymphocytic infiltration, pattern of growth, degree of differentiation, mucinous pattern, age \(\geq 65\) years, and gender). Three significant factors affecting overall survival and disease free survival were selected in a stepwise fashion in increasing order as follows: TNM stage, NK cell infiltration, and lymphocytic infiltration. TNM\textsuperscript{23} stage and peritumoral lymphocytic infiltration\textsuperscript{10,11,12,24,25} have been related to prognosis in colorectal carcinoma in some studies using multivariate analysis. However, although the NK cell infiltration has previously been studied in histopathologic material of colorectal carcinomas\textsuperscript{16,19} to the authors’ knowledge this is the first time that it has been correlated with survival of patients.

In multivariate analysis of patients with Stage III tumors, the number of involved lymph nodes was the first variable entered in the model. After that, the \(P\) value of lymphocytic infiltration increased from 0.001 to 0.008 in overall survival and from 0.006 to 0.018 in disease free survival, appearing as a weak “close alternative” to NK cell infiltration. After NK enters in the second step, lymphocytic infiltration becomes nonsignificant (\(P\) value increased from 0.008 to 0.08 in overall survival and from 0.018 to 0.124 in disease free survival). Lymphocytic infiltration is a clear “close alternative” to NK cell infiltration as prognostic factor. The reason for that is the possible relationship between these two variables.

However when the direct relationship between lymphoid reaction and NK cell infiltration was analyzed, no correlation was found between NK cell number and the degree of lymphocytic infiltration. For example, of 25 patients with extensive NK cell infiltration, 10 (40\%) were found to have moderate or little/none lymphocytic infiltration and 47 of 132 patients with little or moderate NK cell infiltration (35.6\%) showed marked lymphocytic infiltration. This lack of correlation of one to the other is surprising. Although the reason for it is unclear, it may be related to different immunostimulating factors acting on the different subset of lymphoid cells present in colorectal carcinomas.

Another type of lymphoid infiltration, “Crohn’s-like lymphoid reaction” also has been described as a prognostic indicator in colorectal carcinoma\textsuperscript{24–26} The authors found this type of lymphocytic infiltrates in only five cases, but this number of patients was too small to establish any correlation with NK cell infiltration and to statistically analyze patient survival.

Therefore, in the authors’ multivariate model, TNM stage and the number of metastatic lymph nodes were the main prognostic variables in colorectal carcinoma. However, the NK cell intratumoral infiltration and the peritumoral lymphocytic infiltration are also independent prognosticators of survival complementary to tumor invasion and lymph node metastasis.

In conclusion, the authors believe that in colorectal carcinoma, an extensive intratumoral infiltration of NK cells (> three NK cells/mm\textsuperscript{2}) that corresponds to 17 NK cells/mm\textsuperscript{2}, is associated with a favorable tumoral outcome and that NK cell intratumoral infiltration can be used as a variable with prognostic
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