Advances in Brief

CD8+ T Cells Infiltrated within Cancer Cell Nests as a Prognostic Factor in Human Colorectal Cancer

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Abstract

The pathophysiological significance of tumor infiltrating lymphocytes remains controversial. To clarify their role, we performed clinicopathological analysis of CD8+ T cells in 131 cases of human colorectal cancer. CD8+ T cells were classified into three groups by their localization: (a) those infiltrated within cancer cell nests; (b) those distributed in the cancer stroma; and (c) those present along the invasive margin (tumor-host interface). Of these, CD8+ T cells within cancer cell nests were most significantly associated with a better survival of patients by both mono- and multivariate analyses. The impact on survival was similar to that of Dukes' staging. Granzyme B+ cytoplasmic granules were detected in lymphocytes within cancer cell nests, confirming their activated, cytotoxic phenotype. CD8 and Ki-67 double immunohistochemistry confirmed higher proliferative activity of CD8+ T cells within cancer cell nests. Our data suggested that human colorectal cancer tissue was infiltrated by various numbers of T cells that had cytotoxic phenotype, contributing to a better survival of patients. This infiltration of colorectal cancer cell nests by CD8+ T cells could be a novel prognostic factor.

Introduction

Human cancer tissue is infiltrated by TILs2 (1–3). TILs have been considered to be a manifestation of host immune reactions to cancer cells (4). However, its pathophysiological significance in human cancer tissue has remained controversial. One of the major reasons for this was a premise that human cancer arises through evading the host immune surveillance, either as a result of weak immunogenicity of tumor cells or by certain immunosuppressive effects from tumor cells (5–8).

We previously studied host immune responses in EBV-associated gastric cancer, revealing that infiltration of the tumor by CD8+ T cells was the most prominent reaction characterizing this type of cancer compared with usual gastric cancer (9). Infiltrating CD8+ T cells in EBV-associated gastric cancer possessed significantly higher levels of proliferative activity and perforin granules, suggesting their immunologically activated state. These conspicuous reactions could be induced by immunological recognition of certain antigen(s) associated with EBV (9). This study has taught us that CD8+ T cells infiltrated into cancer cell nests could be representative of host immune reactions against cancer cell growth. In the present study, we expanded this concept to human colorectal cancers, which are one of the most common malignancies in the world. In human colon or rectum cancer, previous reports described the following immune-related prognostic factors: (a) continuous lymphocytic reactions along the invasive margin (10); (b) Crohn's-like lymphoid aggregates (11, 12); (c) TILs along the invasive margin and in the stroma (3); and (d) follicular and paracortical (T-zone) hyperplasia in regional lymph nodes (13). These studies suggest that colorectal cancers represent tumors in which certain immune reactions can work to diminish the aggressiveness of cancer cells, despite presumptive immunosuppressive environments. However, the impact of these factors may not be so high, or the assessment method is not so specific from the immunological viewpoint because they were based on conventional histology. We need more simple and reliable methods to assess the host immune reactions. Based on our previous study on EBV-associated gastric cancer, we show here that infiltration of CD8+ T cells within cancer cell nests is a new, reliable prognostic indicator in human colorectal cancer, bearing a similar impact as that of Dukes' staging.

Materials and Methods

Tissue Samples. One hundred thirty-one surgically resected cases of colorectal cancer were randomly selected from the files of Tohoku Rosai Hospital (colon cancer) and Department of Surgery I, Tohoku University Hospital (rectum cancer) with operations performed during 1986 to 1989. These cases had follow-up data for at least 5 years. No cases received radiation or chemotherapy before operation. All cases were histopathologically classified into well, moderately, or poorly differentiated adenocarcinomas according to the WHO classification (14). To stage cancer, we adopted Dukes' classification: A, cancer invasion confined within the submucosa or the muscularis propria without metastasis; B, cancer invading into the subserosa or the adventitia without metastasis; C, with lymph node metastasis; and D, with simultaneous hematogenous metastasis. The number of cases of each stage was 21, 48, 54, and 8 for Dukes' A, B, C, and D, respectively. Metastasis in lymph nodes was checked by histopathological examination in all cases. The approximate number of lymph nodes examined was 10–20 per one case. Liver metastasis was diagnosed either by histopathological examination of metastatic foci or by computed tomography.

Immunohistochemistry. A biotin-streptavidin-peroxidase method using Histofine kit (Nichirei, Tokyo, Japan) was adopted on formalin-fixed, paraffin-embedded sections as recommended by the manufacturer. The primary antibodies used were mouse monoclonal anti-CD8 (clone C8/144B; DAKO, Glostrup, Denmark; 1:100) and anti-granzyme B (clone GrB-7; Kamiya Bio-Medical Co., Seattle, WA; 1:40). The pretreatment condition of specimens was autoclave heating (120°C for 5 min) and microwave heating (95°C for 15 min) for CD8 and granzyme B, respectively. The positive control of granzyme B staining was a case of natural killer cell lymphoma. We counted cells positive for granzyme B with a sparsely granulated pattern as activated cytotoxic T cells using an oil-immersion lens (×1000) in representative 23 cases. Three areas were chosen in each case. Cells strongly positive for granzyme B were excluded because we judged them to be natural killer cells.

Classification of CD8+ T Cells by Location and Their Quantification. By immunohistochemistry for CD8, we classified CD8+ T cells into three groups: (a) those distributed along the invasive margin of cancer; (b) those infiltrated in cancer stroma; and (c) those infiltrated within cancer cell nests (Fig. 1A). For (a) and (b), we semiquantitatively scored the degrees of infiltration into four groups: 0, nil; I, mild; II, moderate; and III, severe. For CD8+ T cells within cancer cell nests, we counted the number of immunoreactive cells with a microscopic field of ×200 (0.933 mm2). Three areas with most abundant distribution were selected, and the average numbers of 0, 1–19,
CDS* T CELLS IN CANCER CELL NESTS

20–49, and over 50 were scored as 0, I, II, and III, respectively. For semi-
quantification of CD8* T cells in cancer stroma, 17 cases were not included
because it was difficult to distinguish CD8* T cells in cancer stroma from
those along invasive margin in those 17 cases.

Statistical Analysis. We quantified or semiquantified each variable as
described above and then made correlation with the patients' survival by
Kaplan-Meier method for each variable (monovariate analysis) using computer
software Stata (Stata Corp., College Station, TX). We judged the difference as
significant when both log-rank and generalized Wilcoxon tests were signifi-
cant. For multivariate analysis, we adopted proportional hazards model (Cox)
and logistic model (Stata).

Correlation between CD8* T Cells within Cancer Cell Nests and
Dukes' Staging. All cases were scored into 0, I, II, or III by the degree of
CD8* T cells within cancer cell nests as described above and into Dukes' A,
B, C, or D. The correlation between the two was tested by Spearman's test
(Stata).

Double Immunohistochemistry (Performed in Representative 15
Cases). Double immunohistochemical analysis for CD8 and Ki-67 was per-
formed as described previously with a modification (9, 15). After autoclave
pretreatment, anti-CD8 monoclonal antibody was applied for 60 min. Histofine
kit for alkaline phosphatase was used with a chromogen new fuchsin (red). To
denature the antibody used, specimens were soaked in boiled water for 10 min.
Mouse monoclonal antibody for proliferating cells (clone Ki-67; DAKO; 1:20)
was applied overnight and then reacted with Envision (K1490; DAKO). The
chromogen was 3,3'-diaminobenzidine tetrahydrochloride (brown). The me-
dian of the labeling index for Ki-67 of CD8* T cells within cancer cell nests
was compared with that of CD8* T cells along the invasive margin by
Mann-Whitney's U test (Stata).

Results

Immunohistochemistry. CD8* T cells were distributed mainly
along the invasive margin and in the stroma. This distribution pattern
was similar to that of lymphocytes by conventional H&E stain. CD8* T
cells were also detected among cancer cells in 75 of 131 cases,
which were scored as I, II, and III, as described in "Materials and
Methods" (Fig. 1B). These cells were designated as CD8* T cells
within cancer cell nests.

Granzyme B* lymphocytes were detected within cancer cell nests
(Fig. 1C) and also in the stroma (data not shown). The average ratio
of lymphocytes positive for granzyme B among CD8* T cells within
cancer cell nests was 28% in 23 cases classified as score III. This
suggests that a part of CD8* T cells within cancer cell nests shows
activated, cytotoxic phenotype. We analyzed the pathophysiological

Table 1 Statistical analysis among CD8* T cells at different localizations
Note that only CD8* T cells within cancer cell nests has a significant impact on the
patients' survival by multivariate analysis. All variables were scored into four groups (see
"Materials and Methods" for details).

<table>
<thead>
<tr>
<th>Variable</th>
<th>Kaplan-Meier P</th>
<th>Proportional hazards model (Cox)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD8* T cells along invasive margin</td>
<td>0.21</td>
<td>0.91 0.68</td>
</tr>
<tr>
<td>CD8* T cells in cancer stroma</td>
<td>0.014</td>
<td>0.81 0.56</td>
</tr>
<tr>
<td>CD8* T cells within cancer cell nests</td>
<td>0.0003</td>
<td>0.52 0.016</td>
</tr>
</tbody>
</table>

* Log-rank test.
**Correlation between CD8⁺ T Cells in Cancer Cell Nests and Dukes' Stage**

In the present report, we analyzed TILs among different localization patterns to demonstrate, for the first time, that CD8⁺ T cells infiltrated within cancer cell nests can be a prognostic factor. The impact of this factor is similar to that of Dukes' staging. The effects by CD8⁺ T cells within cancer cell nests could be theoretically related to the effector function of activated killer T cells. The occurrence of granzyme B⁺ cells suggests that part of CD8⁺ T cells within cancer cell nests are activated CTLs (16). Stimulation of naive T cells is required to induce their proliferation and differentiation into activated T cells. The second signal by the costimulatory molecules is required for this process (17). For the demonstration of this activation mechanism, we have already reported that costimulatory molecules B7-1 and B7-2 are expressed on macrophages distributed along the invasive margin of colon cancer, where T cells are colonized (15). Close localization of cancer cells and B7⁺ macrophages suggests an occurrence of antigen presentation in which certain tumor antigens may be recognized by CD8⁺ T cells within cancer cell nests (15). After being activated with the costimulatory function, T cells may migrate into cancer cell nests, exhibiting a higher proliferation activity.

**Table 3** Correlation between CD8⁺ T cells within cancer cell nests and Dukes' staging

<table>
<thead>
<tr>
<th>Dukes' stage</th>
<th>Score of CD8⁺ T cells within cancer cell nests</th>
</tr>
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<tbody>
<tr>
<td>A</td>
<td>1</td>
</tr>
<tr>
<td>B</td>
<td>20</td>
</tr>
<tr>
<td>C</td>
<td>28</td>
</tr>
<tr>
<td>D</td>
<td>7</td>
</tr>
<tr>
<td>Total</td>
<td>56</td>
</tr>
</tbody>
</table>

**Discussion**

In the present report, we analyzed TILs among different localization patterns to demonstrate, for the first time, that CD8⁺ T cells infiltrated within cancer cell nests can be a prognostic factor. The impact of this factor is similar to that of Dukes' staging. The effects by CD8⁺ T cells within cancer cell nests could be theoretically related to the effector function of activated killer T cells. The occurrence of granzyme B⁺ cells suggests that part of CD8⁺ T cells within cancer cell nests are activated CTLs (16). Stimulation of naive T cells is required to induce their proliferation and differentiation into activated T cells. The second signal by the costimulatory molecules is required for this process (17). For the demonstration of this activation mechanism, we have already reported that costimulatory molecules B7-1 and B7-2 are expressed on macrophages distributed along the invasive margin of colon cancer, where T cells are colonized (15). Close localization of cancer cells and B7⁺ macrophages suggests an occurrence of antigen presentation in which certain tumor antigens may be included (15). After being activated with the costimulatory function, T cells may migrate into cancer cell nests, exhibiting a higher proliferation activity.

**Table 3** Correlation between CD8⁺ T cells within cancer cell nests and Dukes' staging

The figures in the table represent case number. Correlation coefficient, \(-0.38; P < 0.001\).
MHC class I molecule is required to be expressed by cancer cells for the recognition of cancer cells by T cells. In colorectal cancer, cancer cells usually express MHC class I molecules (18). In cervical neoplasia, no clear correlation was reported between the expression of MHC class I molecules by neoplastic cells and infiltration of CD8+ T cells into the neoplastic tissue (19).

Occurrence of metachronous metastasis in the liver or in the lung via a hematogenous route is one of the major causes of death in patients with colorectal cancer. Considering this, our data on CD8+ T cells suggest that these T cells may function not only locally but systemically in the liver and lung as well to suppress micrometastases after being activated in the cancer tissue. On the possible local effects, degeneration of tumor cells was reported to be associated with invasion of cancer tissue by CD4+ T cells and CD11c+ macrophages (18). The mechanism for this finding may be different from the effect of CD8+ T cells observed in this study. We did not observe apparent findings of cancer cell degeneration or cell death in carcinoma nests infiltrated by CD8+ T cells.

There was significant correlation between the degree of CD8+ T-cell infiltration within cancer cell nest and Dukes’ staging, with both factors bearing significant impact on the patients’ survival. This not only suggests a cooperative function by both factors but also indicates that immune reactions, if present, could confine cancer stages to earlier ones by diminishing the aggressiveness of cancer cells.

We dealt with CD8+ T cells in the present study. As discussed above, the interactions are expected between T cells and macrophages. We dealt with all immune/inflammatory cells as a whole in the present study for comparison to CD8+ T cells. We need to further analyze the possible antitumor immunity. If present, could confine cancer stages to earlier ones by diminishing the aggressiveness of cancer cells.

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Acknowledgments

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References

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