Immunological study of the mechanism of action of interleukin-10 on colorectal cancer

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Summary

Purpose: Ever since interleukin-10 (IL-10) was identified as a factor that inhibits cytokine production, it has been assumed to be an accelerator of the progression of colorectal cancer. However, recent information has shown that IL-10 is also involved in activation of the body’s defense mechanisms and immunity. This study was performed with the aim of elucidating the role of IL-10 in colorectal cancer.

Methods: We conducted an immunological study of colorectal cancer tissue and colorectal cancer cell lines.

Results: Immunohistological testing of the colorectal cancer tissue revealed expression of IL-10 in adenocarcinomas that were well and moderately differentiated, while no clear expression was observed in poorly differentiated adenocarcinoma, mucinous adenocarcinoma or signet-ring cell carcinoma. Tests of colorectal cancer-derived glandular epithelial cell lines (HT29, colo320, colo201) revealed predominantly expression of IL-10. Investigation of the effect of the IL-10 produced by tumor cells on lymphocytes showed induction of predominantly toxic activity by IL-10. In addition, expression of Fas ligand (FasL) was induced by addition of human recombinant IL-10 to peripheral T cells in vitro.

Conclusion: The results of this study suggest the possibility that the IL-10 produced by the glandular epithelial cells of colorectal cancer induces FasL molecules by acting on lymphocytes that have infiltrated around the cancer cells, and that it damages the cancer cells via the Fas/FasL system, thus exerting a protective effect on the body.

Background and Purpose of the Study

Humoral factors produced by tumor cells are thought to have an inhibitory effect on the body’s defense responses to tumors and on immune surveillance mechanisms, and they are said to play an important role in tumor progression, but not much is known about their mechanisms of action¹. Interleukin-10 (IL-10) is a factor that was isolated from T cells and identified, and it has been found to be a cytokine that modulates lymphocyte function. Its main actions control the reactivity of T cells to specific antigens and inhibit the biosynthesis of inflammatory cytokines and chemokines secreted by monocytes/macrophages²⁰. Since large amounts of IL-10 have been found in the serum of patients with advanced gastrointestinal cancer in recent years⁶⁰, the possibility that the role of the IL-10 produced by tumor cells includes actions that inhibit proliferation and cytokine production, e.g. TNF production, by T cells that infiltrate tumor tissue, actions that induce the conversion of Th1 cells, which exhibit cytotoxicity, to Th2 cells, and actions that exert inhibitory control on the expression and antigen-presenting ability of MHC class I antigens, which recognize cytotoxic T cells, has been reported with regard to colorectal cancer⁶⁰ as well as basal cell carcinoma.

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squamous cell carcinoma, gliomas, lung cancer, and renal cancer. Moreover, expression of IL-10 by tumor cells has been postulated as a factor that promotes cancer cell progression. Recently, however, new properties of IL-10 have been demonstrated, and actions that increase expression of MHC class II molecules, actions that promote the proliferation and differentiation of B cells and increase antibody production, actions that increase expression of adhesion molecules on vascular endothelial cells and promote lymphocyte infiltration of target organs, and even actions that induce apoptosis and induce cytotoxicity have also been shown, and a contribution to activation of the body's defense mechanisms and immunocompetence has also been reported. Particularly in association with tumors, studies in IL-10 knockout mice have shown much higher incidences of colorectal cancer and enteritis, organ-specifically, than in normal mice, and recovery from these conditions in response to intraperitoneal administration of IL-10 has even been reported in these mice. Thus, properties of IL-10 that are the opposite of the mechanisms of action listed above have also been shown. Moreover, even more recently, clinical trials using IL-10 as a therapeutic agent for a variety of diseases have been instituted, and the many aspects mentioned above of the mechanisms of action of IL-10 on intestinal diseases are still unknown. We therefore used human colorectal cancer tissue and human colorectal cancer cell lines to conduct this study with the objective of elucidating the role of IL-10 in colorectal cancer.

**Materials and Methods**

1. **Immunohistological study**

Surgical specimens obtained from 93 cases of colorectal cancer and stored in the Third Department of Surgery, Tokyo Medical University were used as the material for the immunohistological study. Sections (3 μm) were prepared from the specimens and submitted for immunohistological examination. Some of the sections were stained with hematoxylin and eosin and used to make pathological diagnoses, and histopathological classification was performed according to the Japanese Classification of Colorectal Carcinoma. The sections were placed on silane-coated slides, and in accordance with the routine method, after washing with PBS they were exposed to formic acid for 5 min. Anti-human-IL-10 monoclonal antibody (R&D Systems Inc., Minneapolis, MN) was used to detect IL-10. Anti-keratin antibody (AE3) (Becton Dickinson, Sunnyvale, CA) was used to identify the anti-IL-10 antibody-positive cells, and they were also investigated with anti-CD3, -CD4, -CD8, -CD14, -CD20, -CD21, and HLA-DR antibodies (Coulter Immunotech, Hialeah, FL), anti-factor VIII antibody (Cedarlane Laboratories Limited, Ontario, Canada), anti-FAS antibody (Chemicon International, Inc., Temecula, CA), and anti-FAS-ligand (FasL) antibody (Chemicon International, Inc.). A 1,000-fold dilution of the antibody in PBS was prepared for the antibody reactions, and after applying it to the slides, they were allowed to stand for 1 hour at room temperature. The sections were then washed with PBS and allowed to react with 1,000-fold PBS dilutions of biotinylated anti-mouse IgG or biotinylated anti-rabbit total Ig for 30 min. After washing for 30 min with cold PBS, they were reacted with avidin for 30 min, and avidin-biotin complexes were allowed to form. The sections were again washed with PBS, and the reaction products were colorized by reacting for 5 min with a solution containing 0.005% H2O2 and 0.05% 3,3'-diaminobenzidine in Tris-HCl (0.05 M, pH 7.6). The nuclei were then stained with methyl green. As controls, the same slides were reacted in the same manner with PBS which did not contain mouse IgG, rat total Ig, or the primary antibody.

2. **Cell strains and cell culture**

Colorectal-cancer-derived human cell lines HT 29, colo 320, and colo 201 were supplied by Health Science Research Resources Bank and cultured in a 37°C, 5% CO2 environment in RPMI1640 culture medium (containing 10 mM HEPES, 100 IU/ml penicillin, 100 mg/ml streptomycin) supplemented with fetal calf serum (FCS). The cells were used after stimulation with E. coli-derived lipopolysaccharide (LPS; 10 mg/mL) and interferon-γ (IFN-γ; 10,000 U/ml; Pharmingen, San Diego, CA). Human lymphocyte separation solution (Nacalai Tesque, Inc., Kyoto, Japan) was used to separate T cells from human peripheral blood by the routine method. A neutralization experiment was performed at 0.1 mg/ml using anti-human-IL-10 antibody (PharMingen).

3. **Measurement of FasL by flow cytometry**

After exposing cells to culture fluid to which an MMP inhibitor had been added, various concentrations of recombinant IL-10 (0.001–0.1 ng/mL; Pharmingen) and colorectal cancer cell culture line supernatant were added, and FasL expression on the cell surface was detected with anti-Fas antibody (Chemicon International, Inc.). Mean fluorescence intensity (MFI) was calculated by dividing the measured values for fluorescence intensity with anti-FasL antibody by fluorescence intensity with control antibody. An automated cell analyzer (EPICS, Coulter) was used for the analysis.

4. **Enzyme immunoassay**

An enzyme immunoassay was used to detect IL-10 in the culture supernatant of the colorectal cancer cell lines, and it was performed with an IL-10 ELISA system (Biotrack Ltd, Dorset, UK). Briefly, after adding 50 μl
of culture supernatant to a 96-well microtiter plate and conversion to the solid phase at 4°C, the reaction was allowed to proceed according to the manufacturer’s instructions, and the measurements were made at 492 nm with a spectrophotometer (Titertec Multiscan MC; Flow Laboratories, Corvallis, OR). The data obtained by the ELISA method are expressed as means±SD.

5. Cytotoxicity assays

Colorectal cancer cell line cells (HT29; 2×10⁶) were suspended in 7.5 ml of RPMI-1640 culture medium containing 5% FBS and cultured overnight with 300 μCi of sodium ⁵¹Cr chromate under conditions of 37°C and 5% CO₂. Magnetic beads (2–3×10⁶; Dynal Biotech, Great neck, NY) were used to separate the CD3-positive cells from human peripheral blood. After suspending them in 0.2 ml of RPMI-1640 medium containing 10% FBS, they were stimulated with Con A (EY Laboratories, San Mateo, CA), recombinant human IL-2 (Genzyme Technne, Framingham, MA), recombinant IL-10 (PharMingen) and culture supernatant of the colorectal cancer cell line. Target cells and effector cells were mixed in arbitrary proportions, and the cells were cultured in 200 ml volumes in a 96-well culture plate. After culturing them at 37°C for 4 hours, the cells were centrifuged and radioactivity was measured. The ⁵¹Cr release was evaluated by subtracting the background ⁵¹Cr release by target cells from the ⁵¹Cr release by the experimental group (target cells and effector cells).

Statistical analysis

Statistical analysis was performed using the Mann-Whitney U-test and Student’s t-test for the value of IL-10 (results shown in Fig. 2, 3).

Results

1. Immunohistological study

Histological classification of the hematoxylin and eosin stained sections of the specimens from the 93 cases of colorectal cancer according to the Japanese Classification of Colorectal Carcinoma yielded 62 cases of well differentiated adenocarcinoma, 24 cases of moderately differentiated adenocarcinoma, 2 cases of poorly differentiated adenocarcinoma, 4 cases of mucinous adenocarcinoma, and 1 case of signet-ring cell carcinoma. Immunohistological assessment of IL-10 expression with anti-human-IL-10 antibody revealed clear localization of IL-10 in colorectal cancer epithelial cells in 51 (82.2%) of the 62 cases of well differentiated adenocarcinoma (Fig. 1A, 1B) and in 5 (20.8%) of the 24 cases of moderately differentiated adenocarcinoma, but no clear localization was detected in the cases of poorly differentiated adenocarcinoma, mucinous adenocarcinoma, or signet-ring cell carcinoma (Table 1). Sites that showed localization of IL-10 were also detected in normal glandular epithelial cells adjacent to the tumor tissue in which no evidence of atypia was observed (Fig. 1C). No associations were found between this tendency to express IL-10 and either the sex or age of the patients, nor were any associations with tumor site observed.

Analysis of the lymphocyte tumor infiltration seen in colorectal cancer tissue showed that the degree of cancer tissue infiltration varied with the patient, and no associations were found between the above histological categories and the degree of infiltration. In the cases that exhibited marked infiltration, T cells were found to predominate in the ratios of CD3 cells (Fig. 1D) to B cells that expressed CD20 (Fig. 1E) and CD21. The CD4/CD8 ratios were about the same. Anti-FasL-antibody- (Fig. 1F) and anti-Fas-antibody-positive cells were also detected among these tumor infiltrating lymphocytes, and the anti-Fas-antibody-positive cells were not only clearly localized among the tumor infiltrating lymphocytes, but among the non-cancer epithelial cells as well (Fig. 1G).

<table>
<thead>
<tr>
<th>Table 1</th>
<th>The rate of localization of IL-10 in cancer epithelial cells of the specimens from the 93 cases of colorectal cancer</th>
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<tr>
<td>Histological classification</td>
<td>Localization of IL-10</td>
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<tr>
<td>Well differentiated adenocarcinoma</td>
<td>51/62</td>
</tr>
<tr>
<td>Moderately differentiated adenocarcinoma</td>
<td>5/24</td>
</tr>
<tr>
<td>Poorly differentiated adenocarcinoma</td>
<td>0/ 2</td>
</tr>
<tr>
<td>Mucinous adenocarcinoma</td>
<td>0/ 4</td>
</tr>
<tr>
<td>signet-ring cell carcinoma</td>
<td>0/ 1</td>
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Fig. 1 Immunohistological study of colorectal cancer.
A, B: expression of IL-10 in colorectal cancer and localization of IL-10 in well differentiated adenocarcinoma.
C: localization of IL-10 in normal colonic glandular epithelium.
D: CD3 expressing T cells in colorectal cancer tissue.
E: CD20 expressing B cells in colorectal cancer tissue.
F: anti-FasL antibody-positive cells in colorectal cancer tissue.
G: anti-Fas antibody-positive cells in colorectal cancer tissue.
2. Production of IL-10 by colorectal cancer cells

We used colorectal-cancer-derived epithelial cell lines (HT29, colo320, colo201) as a means of elucidating the ability of tumor cells to produce IL-10 and detected expression of IL-10 in their culture supernatant by ELISA. Expression of IL-10 by non-stimulated cells was also detected, but it was significantly increased by LPS stimulation and IFN-γ stimulation, and the observation of a dose-dependent increase in the amount produced by HT29 cells in response to INF-γ and LPS was a particularly interesting finding (Fig. 2A, B, C).

3. IL-10-mediated cytotoxicity

In order to assess the effect of the IL-10 produced by tumor cells on lymphocytes, its cytotoxicity was evaluated using colorectal cancer line cells (HT29) as target cells and human peripheral blood T cells as effector cells, and allowing IL-10 to act on them. The results clearly showed that significant toxicity was induced by the
addition of IL-10, and its degree of activity was observed to be the same as that of Con A and IL-2, whose ability to induce cytotoxicity has been demonstrated in the past. Moreover, their activity was increased synergistically by the addition of IL-10 to Con A and IL-2 (Fig. 3A). Similar cytotoxicity was detected when the culture supernatant of a colorectal cancer cell line (HT29) stimulated with 10-fold diluted LPS (1.0 μg/ml) was added, and its activity tended to be decreased by anti-IL-10 neutralizing antibody (Fig. 3B).

4. Induction of FasL expression by the culture supernatant of colorectal cancer line cells
Since the above results suggested toxicity due to IL-10 in the culture supernatant of colorectal cancer line cells, and increased FasL expression in response to IL-10 was reported in a recent study in which mouse T cells were used[23], we investigated the ability of IL-10 to induce FasL expression by adding human recombinant IL-10 to human peripheral blood T cells in vitro as a preliminary experiment. The results showed concentration-dependent increases in expression in response to human recombinant IL-10 (Fig. 4A). Next we used logarithmic dilutions of the supernatant as a means of detecting ability to induce FasL expression in the culture supernatant of the colorectal cancer line. The results showed induction of FasL expression that was associated with the dilution rate (Fig. 4B), and the FasL expression was decreased by the addition of anti-IL-10 neutralizing antibody (Fig. 4B).

Discussion
Studies on IL-10 produced by tumor cells have often focused on gliomas[19], lung cancer[20], etc., but have done nothing more than detect expression of IL-10 histologically, and there have been few reports of detailed analyses of its role in cancer tissue. Kim et al.[9] and
Nakagomi et al.\textsuperscript{12} conducted analyses using lymphocytes infiltrating around cancer cells that express IL-10 and detected decreased lymphocyte proliferation capacity and impaired ability to express the various cytokines that tumor-infiltrating lymphocytes express, but no studies directly investigating the effect of IL-10 on tumor cells have ever been reported. The cytotoxicity assays with colorectal cancer cells as the target cells conducted in the present study showed clear toxicity by IL-10, similar in degree to the toxicity displayed by Con A and IL-2, which are known to activate T cells. These findings seemed to imply that human peripheral blood T cells stimulated with IL-10 possess activity that damages colorectal cancer cells, and when we assessed cytotoxicity in a similar manner using the culture supernatant of a colorectal cancer line (HT29), it displayed the same degree of activity. When anti-IL-10 neutralizing antibody was added to determine whether this activity was dependent on IL-10, it was found to decrease approximately 50%, and the results seemed to suggest that the cytotoxicity in the culture supernatant of the colorectal cancer line may not be attributable IL-10 alone, but be due to interactions with other humoral factors that possess toxic activity. The ability of colorectal cancer tumor cells to express cytokines that activate T cells, such as IL-6, has been known for some time\textsuperscript{6,24}, and the results of the present study suggest that colorectal cancer cells produce these cytokines in addition to IL-10.

Perforin/granzyme and the Fas/FasL system have been reported to be involved in the principal mechanisms of cytotoxicity, but their specific individual actions depend on the nature of the target cells\textsuperscript{20}. On the other hand, the majority of perforin-mediated cytotoxicity is by CD8 T cells, and CD4 T cells and NK cells have been found to be cells that use FasL as the effector molecule\textsuperscript{26,27}. In addition, since CD4 T cells, even
Th0, Th1, and sometimes Th2 cells, have been reported to exhibit Fas/FasL-mediated cytotoxicity\textsuperscript{26,27}, CD4 T cells appeared to be largely responsible for the toxicity in colorectal cancer tissue. Since all lymphocytes were tested in the cytotoxicity assays performed in the present study, without separating them into CD8 T cells, CD4 T cells, and NK cells, the actual effector cells are unknown, and we hope to clearly identify them in a future study. Furthermore, large amounts of IL-10 have been detected in the serum of patients with advanced gastrointestinal cancer\textsuperscript{26,27}, and the results of this study suggest that the source of the IL-10 in their serum is the tumor cells.

The immunohistological study of colorectal cancer tissue in this study revealed expression of IL-10 in well and moderately differentiated adenocarcinoma epithelial cells, whereas the rate of expression was low in poorly differentiated adenocarcinoma, mucinous adenocarcinoma and signet-ring cell carcinoma, suggesting that in tumors with a low degree of malignancy tumor cells may be damaged by the IL-10-mediated Fas/FasL system and be readily eliminated from the body. This conclusion partly differs from those of other investigators, and when judged as a whole, it appears that there might be two aspects to the action of IL-10 on tumor cells: a promoting action and an inhibitory action. It will be necessary to conduct a study on the relationship between expression of IL-10 and outcome in the future.

Conclusions

The results of this study suggest that IL-10 produced by glandular epithelial cells of colorectal cancer may eliminate cancer cells through the Fas/FasL system mediated by the lymphocytes that infiltrate around the cancer cells and that IL-10 acts in a manner that defends the body.

Acknowledgements

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大腸癌に対する Interleukin-10 の作用機序に関する免疫学的検討

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【要旨】 IL-10 はサイトカイン産生を抑制する因子として同定されて以来、大腸癌の患者血清中にも多量の IL-10 が検出されるなどの報告から、腫瘍組織内に浸潤する T 細胞の活性化を抑制する可能性などが指摘され、大腸癌進展のための促進因子の一つとして考えられてきた。しかしながら、最近の報告では IL-10 は生体の防御機転や免疫能の賦活化に関与する事も示されており、このことから、本研究では大腸癌における IL-10 の役割を明らかにする目的で、ヒト大腸癌組織（以下、大腸癌組織）ならびにヒト大腸癌細胞株（以下、大腸癌細胞株）を用い解析を行った。

大腸癌細胞を用いた免疫組織学的検討では、IL-10 の発現は比較的分化的程度の高い癌細胞で検出された。更に腫瘍細胞からの IL-10 産生能を明らかにする目的から、大腸癌由来の胸腺皮細胞株（HT29, colo 320, colo 201）を用い検討したところ、優位に IL-10 の発現を検出した。腫瘍細胞から産生される IL-10 のリンパ球に与える影響を検討した結果では、IL-10 による腫瘍組織と癌細胞の生活活性の影響を認め、更に大腸癌細胞株の培養上清中の IL-10 は Fas Ligand (FasL) 発現を誘導することが明らかになった。このことから、大腸癌の癌細胞起源と腫瘍細胞起源のリンパ球に作用し、FasL 分子発現を誘導させ、Fas/FasL システムにより癌細胞の障害に関与する可能性の示唆を示唆された。

(Key words) Interleukin-10, Colorectal cancer, Fas/Fas ligand

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