Regulation of tumor necrosis factor gene expression in colorectal adenocarcinoma: In vivo analysis by in situ hybridization

(Immunohistochemistry)

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ABSTRACT Tumor necrosis factor (TNF) produced by macrophages is thought to contribute to the host defense against development of cancer. However, since tumor cells themselves are able to produce TNF, it is conceivable that TNF may also play an adverse pathologic role in carcinogenesis. To better understand the functional significance of TNF in neoplastic disease, we have determined the cellular source of TNF activity produced in 10 patients with colorectal cancer. Northern blot analysis of RNAs extracted from fresh biopsy specimens revealed detectable TNF mRNA levels in all instances. By using in situ hybridization of frozen sections, scattered cells expressing TNF mRNA could be discerned. Based on morphological criteria, these TNF-positive cells most likely belong to the macrophage lineage. Macrophages in normal tissue surrounding the tumor did not express TNF mRNA, suggesting that macrophage activation occurs locally at the site of neoplastic transformation. Immunohistochemistry using anti-TNF monoclonal antibodies revealed that less than 1% of tumor-infiltrating macrophages synthesize TNF protein. Thus we present evidence that in colorectal cancer only a small proportion of tumor-infiltrating macrophages produces TNF, indicating that the microenvironment of the tumor provides adequate, yet suboptimal, conditions for macrophage activation.

Tumor necrosis factor (TNF) has been originally described as a serum activity in lipopolysaccharide-treated mice that is capable of causing hemorrhagic necrosis of animal tumors (1). However, since the availability of highly purified recombinant TNF, evidence has accumulated indicating that TNF has a wide range of biological activities, affecting the growth, differentiation, or function of virtually every cell type investigated (2-5). Although the complexity of TNF activities makes it at present difficult to assign a specific role to TNF in vivo, its direct tumoricidal activity and its role in inflammation are thought to contribute to the host’s defense against tumors. This proposal has been challenged by findings indicating that many cell types, including tumor cells themselves, are able to produce either TNF or lymphotiksin (also called TNF-β) (6-10). Lymphotiksin is a TNF-related cytokine that binds to the same receptor (11) and exerts similar biological activities (12). TNF or lymphotiksin production by tumor cells suggests that these cytokines have a possible pathogenetic role in tumorigenesis. Indeed, the secretion of lymphotiksin by human myeloma cells has been linked to osteoclastic bone destruction and hypercalcemia in patients with myeloma (8). In addition, because of its potent angiogenic activity (13, 14), TNF may stimulate the growth of blood vessels, thereby promoting tumor development. Furthermore, the ability of TNF to induce collagenase synthesis (15) may lead to tissue destruction facilitating the invasive growth of tumors. Thus, demonstration of TNF production in cancer may reflect either protective or pathogenic processes. Clearly, a first step to understanding the pathophysiological role of TNF in cancer would be the identification of the TNF-producing cell type at the actual site of disease. Thus we analyzed samples from 10 patients with colorectal carcinoma. We show that tumor-infiltrating macrophages and not tumor cells present in colorectal adenocarcinoma express TNF mRNA and produce TNF protein.

MATERIALS AND METHODS

RNA Blotting Analysis. Total cellular RNA was extracted from fresh biopsies by the method of Chirgwin et al. (16), 30 μg of RNA was electrophoresed on formaldehyde gels, transferred to nitrocellulose filters, and hybridized to a TNF-specific cDNA probe (an 800-base-pair EcoRI fragment of A42-4) (17) or to an HLA-B7-specific cDNA (18) labeled with 32PdCTP by random priming. After hybridization, filters were washed and exposed to Kodak XAR films by using an intensifying screen.

In Situ Hybridization. In situ hybridization was performed essentially as described by Hogan et al. (19). Freshly isolated biopsies were frozen immediately without any fixation. Specimen blocks were cut at 8 μm in a cryostat at ~20°C. Serial frozen sections were collected on “subbed” slides (20), dried for 10 min at 55°C, and fixed in 4% (wt/vol) paraformaldehyde. To remove proteins possibly associated with mRNA, slides were incubated in 2× SSC (1× SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7.0) for 30 min at 70°C, rinsed in phosphate-buffered saline (PBS, 20 mM sodium phosphate/0.7% NaCl, pH 7.4), and digested with Pronase (0.2 mg/ml) for 10 min at room temperature. Slides were then refixed, acetylated, dehydrated, and finally air-dried.

RNA probes were labeled by using SP6 or T7 RNA polymerases and 35S-labeled UTP as described (21). Template TNF- and HLA-B7-specific DNAs were linearized by digestion with enough restriction enzyme to generate 35S-labeled run-on transcripts 380-1100 bases long. Hybridization was performed at 50°C in a humid chamber. Slides were washed in 50% (vol/vol) formamide/2× SSC followed by digestion with RNase A (20 μg/ml) and three further washes in 50% formamide/2× SSC. For autoradiography, air-dried slides were coated with NTB2 emulsion (Eastman Kodak) and exposed at 4°C for 10 days. Slides were developed and counter-stained with hematoxylin/eosin.

Immunohistochemistry. For immunostaining frozen sections were incubated with monoclonal antibodies as indicated. Monoclonal anti-human TNF antibody 154/6 was a gift to whom reprint requests should be addressed.

Abbreviation: TNF, tumor necrosis factor.

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of G. Trinchieri (Philadelphia). Monoclonal antibody 63D3, recognizing a monocyte-specific antigen (22), was purchased from the American Type Culture Collection. Alkaline phosphatase-anti-alkaline phosphatase complexes were prepared as described by Cordell et al. (23). Sections were counterstained with hematoxylin/eosin.

RESULTS

TNF mRNA Expression in Colorectal Cancer. Tumor biopsy specimens were obtained from 10 patients with colorectal cancer during abdominal surgery. Total RNA was extracted and analyzed for TNF mRNA levels. As shown in Fig. 1, the abundance of TNF mRNA varied depending on the tumor specimen investigated. Longer exposure of the RNA blot revealed that, indeed, TNF mRNA could be detected in all instances (data not shown). The tumor of patient 7 apparently expressed the highest level of TNF mRNA. Control hybridization to a HLA-A,B,C-specific cDNA probe confirmed the integrity of the RNA samples loaded. The different HLA-A,B,C mRNA levels observed most likely reflect variable HLA-A,B,C gene expression by individual tumor tissues since control ethidium bromide staining indicated equivalent quantities of RNA transferred to nitrocellulose filters.

In Vivo TNF mRNA Expression by Tumor-Infiltrating Macrophages. To determine the cellular source of TNF mRNA, in situ hybridization of frozen tumor sections was performed using a 35S-labeled antisense TNF RNA probe. As exemplified by patient 7, the majority of cells have 5-10 grains per cell (Fig. 2a) corresponding to the background staining revealed by hybridization to a 35S-labeled sense HLA-B7 RNA probe (Fig. 2c). Only a few scattered cells had grain counts that were well above background (40-200 grains), which was considered specific hybridization to TNF mRNA. By histomorphological criteria (azurophilic cytoplasm, large lobular nucleus, and dense chromatin), these cells were considered to be of the macrophage/monocyte lineage.

To demonstrate the sensitivity of the in situ hybridization, we used an 35S-labeled antisense HLA-B7 RNA probe. As shown in Fig. 2b, greater than 80% of cells were positive, indicating that most if not all of the cells contained intact mRNA that was accessible for effective hybridization. Similar results were obtained when tumor specimens from nine other patients were analyzed by in situ hybridization (Table 1).

Tumor-Infiltrating Macrophages Produce TNF Protein. Since TNF gene expression is largely regulated at a posttranscriptional level—that is, translation of TNF mRNA and secretion of TNF require additional stimuli (10, 24, 25)—it was important to determine whether TNF mRNA-positive macrophages actually produce TNF protein. As shown in Fig. 3, immunohistochemistry using an anti-TNF monoclonal antibody strongly stained cells with macrophage-like morphology, indicating that tumor-infiltrating macrophages do produce TNF protein in vivo.

Lack of TNF-Producing Macrophages in Normal Tissue Surrounding the Tumor. Normal colorectal tissue of patient 7 adjacent to the site of tumor had strong mononuclear infiltration (Fig. 4a) comparable to that observed in the adenocarcinoma (Fig. 5a). However, neither TNF mRNA nor TNF protein could be detected by in situ hybridization (Fig. 4b) or by immunostaining (data not shown), respectively. This finding indicates that it is the microenvironment of colorectal cancer that provides the stimuli for the regional activation of macrophages.

Activation Status of Tumor-Infiltrating Macrophages. To determine the level of macrophage activation in colorectal cancer in further detail, we compared the extent of tumor
Table 1. \textit{In situ} analysis of TNF production in colorectal cancer

<table>
<thead>
<tr>
<th>Patient</th>
<th>Site of adenocarcinoma</th>
<th>Frequency of infiltrating macrophages, %</th>
<th>TNF mRNA-positive cells, %</th>
<th>TNF-producing cells, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Sigmoid colon</td>
<td>15-25</td>
<td>2</td>
<td>0.2</td>
</tr>
<tr>
<td>2</td>
<td>Sigmoid colon</td>
<td>18-35</td>
<td>3</td>
<td>0.3</td>
</tr>
<tr>
<td>3</td>
<td>Colon</td>
<td>20-30</td>
<td>1.5</td>
<td>0.2</td>
</tr>
<tr>
<td>4</td>
<td>Colon</td>
<td>15-25</td>
<td>2</td>
<td>0.3</td>
</tr>
<tr>
<td>5</td>
<td>Sigmoid colon</td>
<td>30-40</td>
<td>1.5</td>
<td>0.1</td>
</tr>
<tr>
<td>6</td>
<td>Sigmoid colon</td>
<td>25-35</td>
<td>1.5</td>
<td>0.1</td>
</tr>
<tr>
<td>7</td>
<td>Rectum</td>
<td>25-35</td>
<td>6</td>
<td>0.3</td>
</tr>
<tr>
<td>8</td>
<td>Rectum</td>
<td>25-35</td>
<td>2</td>
<td>0.1</td>
</tr>
<tr>
<td>9</td>
<td>Colon</td>
<td>20-30</td>
<td>1</td>
<td>0.1</td>
</tr>
<tr>
<td>10</td>
<td>Rectum</td>
<td>30-40</td>
<td>&lt;1</td>
<td>0</td>
</tr>
</tbody>
</table>

* Cryostat sections of tumor biopsy specimens were immunostained with the monocytic-specific antibody 63D3. Frequencies of infiltrating macrophages were revealed by microscopic evaluation of several visual fields corresponding to more than 1000 nucleated cells. Data are the ranges of monocyte infiltration that varied within a given tumor specimen depending on the particular section investigated.

\textsuperscript{1}TNF mRNA-positive cells were visualized by \textit{in situ} hybridization with an antisense TNF complementary RNA probe. Frequencies of TNF mRNA-positive cells were estimated by evaluating >2000 cells.

\textsuperscript{2}TNF protein-producing cells were detected by immunostaining with anti-TNF antibody. Frequencies of positive cells were estimated by evaluating >2000 cells.

Infiltration by macrophages with the frequency of macrophages expressing TNF mRNA or producing TNF protein. As shown in Fig. 5a, approximately 30% of the cells in the rectal adenocarcinoma of patient 7 had macrophage-like morphology and were stained with 63D3, a monocytic-specific monoclonal antibody (22). Bright- and dark-field microscopic evaluation of the \textit{in situ} hybridization using \textsuperscript{35}S-labeled antisense TNF RNA probe showed that 6% of the cells were TNF mRNA-positive (Fig. 5c and d). When immunostained by the anti-TNF monoclonal antibody, only 0.3% of the mononuclear cells were positive (Fig. 5b and Table 1). Thus the data indicate that approximately every fifth tumor-infiltrating macrophage only expresses TNF mRNA and even fewer synthesize detectable levels of TNF protein. Similar results were obtained from corresponding analyses of nine other patients with colorectal adenocarcinoma (Table 1).

\textbf{Fig. 3.} \textit{In vivo} TNF production in rectal adenocarcinoma. Cryostat sections from the adenocarcinoma of patient 7 were immunostained with monoclonal anti-TNF-antibody 154/6 using the alkaline phosphatase-anti-alkaline phosphatase method. Parallel sections analyzed with isotype-matched control immunoglobulins were not stained. (\times 600.)

\textbf{Fig. 4.} Lack of TNF mRNA-positive cells in normal tissue. Cryostat sections of normal rectal tissue from patient 7 in the immediate vicinity of the tumor were immunostained with monocytic-specific monoclonal antibody 63D3 (a) or hybridized to a \textsuperscript{35}S-labeled anti-sense TNF RNA probe (b). Arrows indicate macrophages identified by morphological criteria. (\times 250.)

\textbf{DISCUSSION}

TNF mRNA has been detected (26) in RNA samples extracted from either peripheral blood lymphocytes or solid tumor tissue from cancer patients. Determination of either TNF activity in the serum or TNF mRNA levels in the RNA extracted from heterogenous tumor tissue, however, does not answer the crucial questions as to the cellular source and functional significance of TNF production in neoplastic disease. In the present study, we have attempted to characterize the conditions of TNF production in colorectal cancer. We show by \textit{in situ} hybridization and immunohistochemistry that in all cases of colorectal carcinoma investigated, TNF was produced by infiltrating macrophages. The observation that the mononuclear infiltrate expresses TNF mRNA in 10 out of 10 colorectal carcinoma patients suggests that in this tumor TNF production may be a defense reaction executed by activated macrophages.

One of the principal findings of this report is that only a fraction of tumor-infiltrating macrophages expresses TNF mRNA, and even fewer are able to produce TNF protein. Although it appears to be difficult to precisely judge the sensitivity of the \textit{in situ} hybridization and immunostaining procedures, analogous analyses of renal cell carcinoma specimens have shown that it is possible to demonstrate TNF mRNA as well as TNF protein in greater than 50% of the tumor-infiltrating macrophages (I.W. and M.K., unpublished data). Thus, the frequencies of TNF-positive macrophages observed might be only slightly underestimated. The quantitative representation of tumor-infiltrating macrophages ex-
pressing TNF mRNA or TNF protein detected in this study suggests that the control mechanisms of TNF gene expression defined in vitro are recapitulated in vivo. It has been shown that murine macrophages require a sequence of two distinct signals to become activated for cellular toxicity (27, 28), which may be TNF-mediated (29, 30). The first signal serves as a "priming" factor and the second signal triggers the final activation. The priming signal may mediate transcriptional activation of the TNF gene, and the second signal may be required to allow translation of the protein and TNF secretion by macrophages (24, 25, 31, 32). As illustrated in this report, the number of detectable TNF-producing macrophages is quite small, even though the tumor is heavily infiltrated. This suggests that the population of tumor-infiltrating macrophages is only suboptimally activated. Activation of macrophages apparently occurs at the site of neoplastic transformation, since macrophages present in the normal tissue in the immediate vicinity of the tumor neither express TNF mRNA nor secrete TNF protein. It is therefore concluded that the colorectal tumor itself provides the microenvironment for macrophage activation.

Our findings have implications for entering patients with colorectal cancer into clinical trials with biological response modifiers. Since, in this tumor, the majority of infiltrating macrophages seems to be suboptimally activated, treatment with recombinant TNF may supplement the host's defense against tumor progression and eventually benefit the patient. Furthermore, treatment with macrophage-activating agents, such as the interferons, might enhance the endogenous antitumoral activities of the mononuclear infiltrate.

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