Increased Apoptosis in Infiltrating Mononuclear Cells of Colorectal Cancer

A Mechanism for Tumor Escape

George G. Chen, MD, PhD; Janet F. Y. Lee, MD; Ursula P. F. Chan, MPhil; Hu Xu, PhD; Ping C. Ip, BS; Wan Y. Lau, MD

- Context.—Disturbance in apoptosis has been proposed as one of the mechanisms involved in the immune response targeting tumor outgrowth. How colorectal cancer cells escape from attack by the immune system is not yet fully understood.

Objective.—To investigate apoptotic molecules associated with colorectal cancer counterattack.

Design and Setting.—Tissue samples of colon from 12 patients with colorectal cancer were collected and analyzed by immunostaining. In addition to tumorous tissues, corresponding nontumorous specimens of colon were obtained as controls.

- Main Outcome Measures.—We examined the expression of Bcl-2, Bcl-xl, Bax, caspase-3, and inducible nitric oxide synthase in infiltrating mononuclear cells of colorectal cancer tissues and also in colorectal cancer tissues. The TUNEL assay was used to detect in situ apoptosis.

- Results.—Apoptosis was barely detectable in specimens of colorectal cancer, which was consistent with an increase in Bcl-2 level and a decrease in caspase-3 level. In contrast, infiltrating mononuclear cells of tumorous tissues showed a marked increase in apoptosis compared with those of nontumorous tissues. The increased apoptosis might have resulted from an imbalance of antiapoptotic and proapoptotic molecules, as reflected by reduction of Bcl-2 level and elevation of Bax level. The elevated caspase-3 levels found in this study could be a downstream effector of the Bcl-2 and Bax apoptotic pathways. A significant increase in inducible nitric oxide synthase observed in the infiltrating mononuclear cells might contribute to immunosuppression seen in colorectal cancer.

- Conclusion.—It is tempting to speculate that aberrant expression of apoptotic molecules and inducible nitric oxide synthase in infiltrating mononuclear cells provides the underlying mechanism through which colorectal cancer cells escape attack by the immune system and subsequently grow without control.

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Apoptosis is a biologic phenomenon of critical importance in the regulation of cell populations in a number of physiologic and pathologic situations, such as cancer development. Recent studies have demonstrated that human colorectal cancer cells show an increase in proapoptotic molecules, for example, Fas and Fas ligand (FasL). It remains unclear why such cancer cells continue to proliferate rather than become apoptotic. Several mechanisms of resistance against apoptosis in colorectal cancer cells have been proposed. Fas counterattack, also known as the tumor immune privilege, is the best known theory. Fas ligand triggers apoptosis in cells, which express its cell surface receptor, Fas. Fas ligand–mediated apoptosis contributes to regulation of the immune system through its role in tolerance acquisition, by which T-lymphocyte activation induces cell death and immune-response termination. Fas ligand expressed in tumor cells mediates immune privilege by inducing apoptosis in infiltrating Fas-sensitive mononuclear cells and other immune effector cells. Fas ligand has been shown to confer immunological privilege in a number of human cancers, including colon cancer. Very recently, however, a study by Favre-Felix et al rejected this tumor counterattack theory because colon cancer monolayers fail to inhibit the growth of Fas-expressing lymphoid cells.

The immune system plays an important role in the regulation of malignancies in humans. Stimulation of the immune system may contribute to tumor induction. For example, immunosuppressive therapy used for allogeneic organ transplantation is associated with an increased risk of development of cancers. In solid tumors, the presence of tumor-infiltrating mononuclear cells, such as lymphocytes, natural killer cells, and macrophages, provides an effective antitumor response. Evidence shows that the number of tumor-infiltrating mononuclear cells in resected tumor specimens negatively correlates with the size of tumor, but positively correlates with survival in various malignancies, including melanoma, lung cancer, and colon cancer.

Mononuclear cells are known to express apoptotic molecules other than Fas and FasL, such as Bcl-2, Bax, and caspases. However, the roles of these non-Fas/FasL apoptotic molecules in infiltrating mono-
nuclear cells of human colorectal cancer have not yet been studied. Therefore, the aims of the present study were to examine non-Fas/FasL apoptotic pathways in infiltrating mononuclear cells of colorectal cancer and to test whether there is a novel mechanism contributing to the growth of human colorectal cancer. The expression of Bcl-2, Bax, and caspases was also analyzed in colorectal cancer of both tumors and nontumorous tissues.

MATERIALS AND METHODS

Specimens

Tissue samples of colon from 12 patients with colorectal cancer were analyzed. In addition to tumors, tissues corresponding nontumorous colon tissues were obtained as controls. Prior to surgery, we obtained informed consent from all patients for subsequent use of their resected tissues. All specimens were immediately frozen after surgical resection and stored in liquid nitrogen before immunohistochemical analysis. All tumors and nontumorous tissue specimens were confirmed by pathologic examinations.

Immunostaining

Tissues stored in liquid nitrogen were thawed at room temperature. After being thawed, they were fixed in 10% neutral buffered formalin solution, processed, and embedded in paraffin. Formalin fixation before embedding was less than 30 hours throughout, which is important for preserving the antigenic determinants analyzed in this study. Tissue specimens were sectioned at 4 μm. Immunostaining was performed on paraffin sections according to the standard procedure described in the ABC kit from Vector Laboratories (Burlingame, Calif). In brief, tissue sections were deparaffinized and rehydrated through 3 changes of xylene and graded alcohol. Tissue sections were then boiled in citrate-based antigen unmasking solution for 1 minute and cooled in Milli-Q water; the endogenous peroxidase activity in the sections was subsequently quenched in 3% hydrogen peroxide solution for 5 minutes. Normal blocking serum (1.5%) supplemented with avidin solution (Avidin/Biotin Blocking Kit, Vector) was used to block tissue sections for 30 minutes. Afterward, preparations were incubated with a primary antibody overnight at 4°C. Anti-human Bcl-2, Bcl-xl, Bax, caspase-3, and inducible nitric oxide synthase (iNOS) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, Calif). The primary antibody was prepared in 1.5% normal blocking serum supplemented with biotin solution from the Avidin/Biotin Blocking Kit and was used at a working dilution of 1:200. After tissue sections were washed with phosphate-buffered saline (PBS), a biotinylated secondary antibody, immunoglobulin G, was applied for 30 minutes. Tissue sections were then washed with PBS, and ABC reagent conjugated with horseradish peroxidase was applied for 30 minutes. Bcl-2 antigen staining was visualized by NovaRED substrate, and other reactions were visualized by DAB substrate (both from Vector). The reaction was terminated by rinsing the sections in tap water. All incubations were done in a humidified environment at room temperature, except where indicated in the text. Finally, sections were counterstained with Vector Gill hematoxylin. After dehydration through graded alcohol and clearing with xylene, slides were mounted with DPX permanent mountant. Negative controls were prepared by replacing the primary antibody with PBS.

Immunoreactivity for each antigen in tissues was examined with an Eclipse TE300 microscope (Nikon, Tokyo, Japan) equipped with a 3CCD camera (DC-330, DAGE-MTI, Michigan City, Ind). The image was analyzed by MetaMorph Imaging System (Universal Imaging Corporation, Pa), and scores were assigned according to the standard described in Table 1.

Table 1. Scoring Standard for Immunostaining

<table>
<thead>
<tr>
<th>Grade</th>
<th>Scoring by Tissue</th>
<th>Scoring by Cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>1</td>
<td>Slight</td>
<td>Part of the cytoplasm</td>
</tr>
<tr>
<td>2</td>
<td>Moderate</td>
<td>Whole cytoplasm without reticular deposits</td>
</tr>
<tr>
<td>3</td>
<td>Strong</td>
<td>Reticular deposits in &lt;1/3 of cytoplasm</td>
</tr>
<tr>
<td>4</td>
<td>Extremely strong</td>
<td>Reticular deposits in ≥1/3 of cytoplasm</td>
</tr>
</tbody>
</table>

Table 2. Immunostaining of Colorectal Cancer Specimens

<table>
<thead>
<tr>
<th>Markers</th>
<th>Tissue Source</th>
<th>No. of Specimens</th>
<th>Score</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bcl-2</td>
<td>Tumor</td>
<td>12</td>
<td>2.67 ± 0.14</td>
<td>.01</td>
</tr>
<tr>
<td>Bcl-xl</td>
<td>Nontumor</td>
<td>12</td>
<td>1.92 ± 0.26</td>
<td>.49</td>
</tr>
<tr>
<td>Bax</td>
<td>Tumor</td>
<td>12</td>
<td>3.45 ± 0.25</td>
<td>.01</td>
</tr>
<tr>
<td>Caspase-3</td>
<td>Nontumor</td>
<td>12</td>
<td>2.75 ± 0.28</td>
<td>.04</td>
</tr>
<tr>
<td>Inducible nitric oxide synthase</td>
<td>Tumor</td>
<td>12</td>
<td>3.00 ± 0.21</td>
<td>.04</td>
</tr>
</tbody>
</table>

* Compared with nontumorous group.

RESULTS

Immunoreactivity in Tumorous and Nontumorous Tissues

The immunostaining results for apoptotic molecules and iNOS are presented in Table 2. Positive cytoplasmic immunoreactivity for all antigens examined was detected...
in most tissue samples, both from tumorous and nontumorous areas. The intensity of Bcl-2 expression was significantly increased in a proportion of ductal and acinar colorectal epithelial cells from tumorous tissues (2.67 \pm 0.14), compared with those from nontumorous tissues (1.92 \pm 0.26, \( P = .01 \)). However, the level of Bcl-xl, another antiapoptotic member of the Bcl-2 family, did not differ between tumorous and nontumorous specimens. To our surprise, colorectal epithelial cells from tumorous tissues expressed a significantly high level of Bax, a proapoptotic molecule that counteracts the Bcl-2 function by homodimerizing or heterodimerizing with Bcl-2.16 Expression of iNOS was also much higher in tumorous areas than in nontumorous ones (3.00 \pm 0.21 vs 1.96 \pm 0.26, \( P = .04 \)). We further investigated the expression of caspase-3, one of the key executors of apoptosis. The reason for studying caspase-3 was that caspase-3 is responsible either partially or totally for the proteolytic cleavage of many key proteins in the pathway of apoptosis related to the Bcl-2 family and iNOS.15 As expected, our experiment showed that the level of caspase-3 was markedly reduced in tumorous tissues (2.75 \pm 0.52) compared with nontumorous tissues (3.64 \pm 0.14, \( P = .04 \)).

### Immunoreactivity in Mononuclear Cells

Infiltrating mononuclear cells in the human colorectal cancer samples examined in this study consisted of lymphocytes and macrophages; the former were predominant. Results of immunostaining are shown in Table 3. The expression of Bcl-2 was much lower in mononuclear cells located in tumorous tissues than in those in the nontumorous samples (1.59 \pm 0.31 vs 2.55 \pm 0.25, \( P = .008 \)) (Figure 1, A and B), whereas expression of its mammalian homolog, Bcl-xl, did not change significantly between tumorous and nontumorous specimens. The intensities of all other antigens tested were significantly stronger in mononuclear cells of tumorous areas than in those of nontumorous areas (Table 3, Figure 1, C through H). No significant neutrophil infiltration was observed in any of the colorectal cancer tissues examined.

### Apoptotic Index

Apoptosis, as detected by the DeadEnd apoptosis detection kit, was basically not detectable in tumor cells or epithelial cells of both tumorous and nontumorous tissues. However, the mean apoptotic index was significantly higher in infiltrating mononuclear cells of tumorous tissues (10.07 \pm 1.93%) than in those of nontumorous tissues (3.13 \pm 0.52%, \( P = .003 \)). The apoptotic mononuclear cells mainly consisted of lymphocytes (Figure 2). Occasionally, apoptosis in infiltrating macrophages was also observed. Apoptosis was not detectable in endothelial cells of blood vessels and lymphoid vessels.

### Apoptotic Index in Relation to Other Molecules Tested

To study the relationship between the apoptotic index and the apoptotic molecules tested, the data were analyzed by Spearman correlation and linear regression. Results of these calculations are presented in Table 4. The apoptotic index was negatively correlated with the expression of Bcl-2 and was unrelated to the level of Bcl-xl in infiltrating mononuclear cells in colorectal cancer tissues. In contrast, the levels of Bax, caspase-3, and iNOS were positively correlated with the apoptotic index in infiltrating mononuclear cells of tumor specimens.

### COMMENT

In this study, we demonstrated that the expression of several apoptotic or apoptosis-related markers differed between tumorous and nontumorous tissues. Unlike antiapoptotic Bcl-xl, the expression of Bcl-2, a proapoptotic molecule, was significantly increased in tumorous tissues as compared with nontumorous samples from the same subject. Although the level of Bcl-2 expression in colon cancer is still debatable,15,16 our results are in agreement with most studies in this area.17-20 Overexpression of Bcl-2 might be related to a high level of cyclooxygenase-2 (COX-2) in colon cancer, as prostaglandin E2, a metabolite of COX-2, induces Bcl-2 expression and inhibits apoptosis in human colon cancer cells.19 The level of Bax, a conserved homolog of Bcl-2, was also significantly increased in tumorous tissues, which was an unexpected result. Bax and Bcl-2 are a pair of important molecules that control apoptosis, with Bax being a promoter of apoptosis and Bcl-2 a protector of apoptosis. Overexpression of Bcl-2 is known to delay or inhibit Bax conformation change and membrane translocation, which is necessary for Bax to induce apoptosis.16,17 In contrast to Bcl-2, an increase in Bax expression sensitizes tumor cells to apoptosis induced by various agents.16,18 Therefore, the balance between Bcl-2 and Bax may determine whether a cell is susceptible to apoptosis. To our knowledge, expression of Bax has not been investigated in colorectal cancer tissues previously. Obviously, an increase in Bax did not actually promote the death of epithelial cells in the colorectal cancer tissues examined, as apoptosis was basically not detectable by TUNEL assay in tumor tissues in the present study.

The lack of apoptosis in colorectal cancer tissues was also in line with the reduced levels of caspase-3 found in this study. Caspase-3 is one of the key executors of apoptosis in Bcl-2 and Bax pathways.17,18 Therefore, the significance of the increased Bax level in colorectal cancer

### Table 3: Immunostaining of Mononuclear Cells

<table>
<thead>
<tr>
<th>Markers</th>
<th>Cell Location</th>
<th>No.</th>
<th>Score</th>
<th>( P^* )</th>
</tr>
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<tbody>
<tr>
<td>Bcl-2</td>
<td>Tumor</td>
<td>11</td>
<td>1.59  \pm 0.31</td>
<td>.008</td>
</tr>
<tr>
<td></td>
<td>Nontumor</td>
<td>11</td>
<td>2.55  \pm 0.25</td>
<td></td>
</tr>
<tr>
<td>Bcl-xl</td>
<td>Tumor</td>
<td>11</td>
<td>1.91  \pm 0.19</td>
<td>.09</td>
</tr>
<tr>
<td></td>
<td>Nontumor</td>
<td>11</td>
<td>2.64  \pm 0.20</td>
<td></td>
</tr>
<tr>
<td>Bax</td>
<td>Tumor</td>
<td>11</td>
<td>2.55  \pm 0.31</td>
<td>.002</td>
</tr>
<tr>
<td></td>
<td>Nontumor</td>
<td>11</td>
<td>1.41  \pm 0.27</td>
<td></td>
</tr>
<tr>
<td>Inducible nitric oxide synthase</td>
<td>Tumor</td>
<td>11</td>
<td>3.27  \pm 0.20</td>
<td>.002</td>
</tr>
<tr>
<td>Caspase-3</td>
<td>Tumor</td>
<td>11</td>
<td>2.23  \pm 0.16</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Nontumor</td>
<td>11</td>
<td>1.68  \pm 0.22</td>
<td></td>
</tr>
</tbody>
</table>

* Compared with mononuclear cells from nontumorous group.

### Table 4: Correlation Between Apoptotic Index and Apoptosis and Apoptotic or Apoptosis-related Molecules in Mononuclear Cells

<table>
<thead>
<tr>
<th>Molecule</th>
<th>( r )</th>
<th>( F )</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bcl-2</td>
<td>-0.6685</td>
<td>7.2702</td>
<td>.02</td>
</tr>
<tr>
<td>Bcl-xl</td>
<td>0.6010</td>
<td>4.5234</td>
<td>.07</td>
</tr>
<tr>
<td>Bax</td>
<td>0.6488</td>
<td>6.5429</td>
<td>.03</td>
</tr>
<tr>
<td>Inducible nitric oxide synthase</td>
<td>0.6438</td>
<td>6.3707</td>
<td>.03</td>
</tr>
<tr>
<td>Caspase-3</td>
<td>0.7724</td>
<td>13.309</td>
<td>.005</td>
</tr>
</tbody>
</table>

Apoptosis in Mononuclear Cells of Colorectal Cancer—Chen et al
Figure 1. Expression of Bcl-2, Bax, caspase-3, and inducible nitric oxide synthase (iNOS) in infiltrating mononuclear cells. Mononuclear cells from tumorous tissues were weakly stained by Bcl-2 antibody (A) (original magnification ×400), while those from nontumorous tissues were strongly reactive with Bcl-2 antibody (B) (original magnification ×400). Immunostaining intensity for Bax was very strong in some of mononuclear cells of tumorous tissues (C). Both cytoplasm and nuclei were positive (original magnification ×400). Mononuclear cells from nontumorous tissues were barely reactive with Bax antibody (D) (original magnification ×400). Epithelial cells of both tumorous (E) (original magnification ×400) and nontumorous tissues (F) (original magnification ×400) were positive for caspase-3, but the intensity was lighter in the former than the latter. In contrast, the staining of mononuclear cells from the former was more intense than those from the latter. Both epithelial cells and mononuclear cells from tumorous tissues were intensely stained by iNOS antibody (G) (original magnification ×400), compared with those from nontumorous tissues (H) (original magnification ×400).
tissues deserves further evaluation. Nevertheless, the balance between Bcl-2 and Bax, or more correctly between cell proliferation and loss of cells by apoptosis, was in favor of the former in human colorectal cancer.

Another significant finding of the present study was that the level of iNOS was significantly elevated in tumorous tissues as compared to nontumorous areas. It has been reported that excessive nitric oxide production by iNOS may contribute to the pathogenesis of colon cancer progression at the transition of colon adenoma to carcinoma in situ.26

Avoiding attack by the immune system is important in neoplastic development. Tumor cells escape immunological rejection using diverse mechanisms. Disturbance of apoptosis has been proposed as one of these mechanisms. To explore the pathway of tumor escape involved in the development of human colorectal cancer, we first demonstrated a significant increase in apoptosis in infiltrating mononuclear cells of colorectal cancer tissues. Furthermore, our results suggested that disturbance of apoptosis resulted from an imbalance of decreased antiapoptotic molecules and increased proapoptotic ones, reflected by a reduction in the Bcl-2 level and elevation of the Bax level. The shift of balance between Bcl-2 and Bax in favor of the latter is known to accelerate the activity of caspase-3, because Bcl-2 expression leads to functional inhibition of caspase-3, whereas Bax promotes caspase-3 activity.17,18 Therefore, caspase-3 can be considered an executor of apoptosis in Bcl-2 and Bax pathways. In line with the role of caspase-3 in apoptosis caused by a decrease in Bcl-2 and an increase in Bax, the expression of caspase-3 was found to be significantly elevated in infiltrating mononuclear cells of colorectal cancer in the present study. The apoptosis or the activity of caspase-3 may also be associated with iNOS,20 whose level was found to increase in the present study. However, conflicting reports have been produced, in which some favor its proapoptotic effect, while others support its antiapoptotic function.27,28 There is also a study suggesting that a low concentration of nitric oxide inhibits apoptosis, whereas a high concentration induces apoptosis.29 Since we were unable to measure the concentration of either nitric oxide or its enzyme, iNOS, in tissues and we also have no standard reference with which to define what is a low or high nitric oxide level, the role of iNOS in apoptosis of mononuclear cells as examined here was uncertain. Another function of iNOS is its role in the regulation of the immune system. This role is particularly important in the present study because mononuclear cells are the main effector in the immune system’s response to tumors. Nitric oxide generated by iNOS influences not only the cytotoxicity of macrophages, but also lymphocyte responses.30,31 Moreover, the inhibition of nitric oxide production revokes immunosuppression and benefits progressively growing malignant tumors.32 Therefore, an increase in iNOS expression in infiltrating mononuclear cells is believed to down-regulate the immune response against colorectal cancer cells and thus may represent an additional effort of colorectal cancer cells to escape the immune system.

Infiltrating mononuclear cells found in colorectal cancer specimens consisted mainly of lymphocytes, with a small number of macrophages. Both lymphocytes and macrophages are well known for their roles in antitumor response.11-15 Tumor immunology consists of 2 essential concepts: immune surveillance, which specifies the host’s immune reactions to tumor cells, and tumor counterattack, which refers to the tumor cell evasion process against the host immune system. Several scenarios have been proposed to be responsible for tumor counterattack mechanisms. One popular scenario suggests that FasL-expressing cancer cells counterattack Fas-expressing tumor-infiltrating lymphocytes and thus escape rejection by the immune system.34 However, this theory is being challenged, as Favre-Felix6 found that colon cancer monolayers did not inhibit the growth of Fas-expressing lymphoid cells. Furthermore, tumor cells transfected with FasL did not enable them to enjoy “tumor escape”; instead, they were rapidly rejected by the immune system.33 Our study suggests that a significant proportion of infiltrating mononuclear cells may lose their immune function by either apoptosis caused by an imbalance of Bcl-2 and Bax or immunosuppression resulting from overexpression of iNOS. Therefore, aberrant expression of apoptotic molecules and iNOS in infiltrating mononuclear cells may be considered mechanisms through which colorectal cancer cells escape the attack of the immune system and subsequently grow without immune control. However, we are unable to define whether this loss of immunological cells in colorectal cancer is a dysfunction of immune surveillance or an effort of tumor counterattack, as pathways leading to the change of apoptotic molecules or iNOS in infiltrating mononuclear cells are still unknown.

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References


