Expression of A Novel Antiapoptosis Gene, Survivin, Correlated with Tumor Cell Apoptosis and p53 Accumulation in Gastric Carcinomas

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Abstract

A novel inhibitor of apoptosis designated survivin has recently been found in many common human cancers but not in normal tissues. A potential distribution of survivin in gastric cancer and its implication for apoptosis inhibition have been investigated. Recombinant survivin expressed in Escherichia coli as a glutathione S-transferase fusion protein was used to raise a novel panel of mouse monoclonal antibodies. In an immunohistochemical analysis of 174 cases of gastric carcinomas (stages I–III), anti-survivin monoclonal antibody 8E2 (IgG1) reacted with 34.5% of cases (60 of 174 cases) with a variable number of tumor cells stained (20–100%). In contrast, no expression of survivin in neighboring normal tissues was observed. When stratified for p53 and bcl-2 expression and apoptotic index, the expression of survivin significantly segregated with p53- and bcl-2-positive cases [56.1 versus 15.2% (P = 0.001) and 69.2 versus 31.6% (P = 0.006), respectively] and with a decreased apoptotic index as compared with that of survivin-negative tumors (0.97 ± 0.64 versus 0.62 ± 0.39%, P < 0.001). These data identify a role for survivin in promoting aberrantly increased cell viability in gastric cancer and suggest a potential correlation between accumulated p53 and survivin expression in neoplasia.

Introduction

For a tumor to become established, compromises in the control of cell proliferation as well as in the control of cell death are necessary (1). In this context, a number of gene products have been potentially implicated in the modulation of cancer cell viability, the resistance to programmed cell death (apoptosis), and the enhancement of tumor progression (1–4). Among the regulators of cell death, IAP3 proteins have recently emerged as modulators of an evolutionarily conserved step in apoptosis, which may potentially involve the direct inhibition of terminal effector caspases 3 and 7 (5). Recently, a novel and structurally unique member of the IAP gene family designated survivin was identified (6). Unlike other IAP proteins, survivin was found during embryonic and fetal development, was completely down-regulated and undetectable in normal adult tissues, and became prominently reexpressed in all of the most common human cancers, including cancers of the lung, colon, pancreas, prostate, and breast, in vivo (6). This selective distribution of survivin in cancer was at variance with the limited expression of other IAPs, most notably bcl-2, in several solid cancers and with its presence in both the normal and transformed cell types (7–9). In this study, we sought to investigate the distribution of survivin in gastric cancer and its potential relationship with p53 and bcl-2 as established modulators of tumor cell progression and viability. We found that the expression of survivin correlated with the presence of both p53 and bcl-2 oncoproteins and translated in drastically reduced apoptosis of cancer cells in vivo.

Materials and Methods

Patients and Samples. The surgically resected specimens used for this study were obtained from consecutive patients with gastric cancer who had undergone potentially curative tumor resection at the Department of General and Gastroenterological Surgery, Osaka Medical College Hospital during the period from 1987–1990. Materials were composed of 48 stage I cases, 52 stage II cases, and 74 stage III cases. Patients who died within 1 month after surgery or died of another disease within 3 months were excluded from this study. Clinical and histopathological examinations were performed in accordance with The General Rules for Gastric Cancer Study (10). The histological types of tumors were reviewed and classified according to Lauren (11) as intestinal or diffuse (including mixed and unclassified), and the disease stage was defined in accordance with the tumor-node-metastasis (TNM) classification (12). There were 124 males and 50 females, and the mean age of the patients was 59.7 years (SD, 12.0 years; range, 20–88 years). Patients had received neither chemotherapy nor radiation therapy before surgery. Formalin-fixed paraffin-embedded blocks of primary tumors were taken from pathological archives. Serial sections of 2–4 µm were prepared from the cut surface of the blocks at the maximum cross-section of the tumor.

Expression of Recombinant Survivin and Generation of Anti-Survivin mAb. A full-length survivin cDNA of 1.6 kb was directionally cloned in frame in the BamHI and EcoRI sites of the prokaryotic expression vector pGEX2T (Pharmacia, Piscataway, NJ) with transformation in the BL21 Escherichia coli strain (Pharmacia). The recombinant survivin was expressed as a GST fusion protein in the presence of 0.1 mM isopropyl-1-thio-β-D-galactopyranoside (Calbiochem, La Jolla, CA) with extraction in 25 mM Tris-HCl, 50 mM glucose, 10 mM EDTA (pH 8.0), and 2 mg/ml lysozyme followed by lysis in 2% Triton X-100 and sonication. The sonicate was centrifuged at 12,000 × g for 10 min at 4°C, and the supernatant applied to a glutathione-Sepharose 4B column for 14 h at 4°C with constant agitation. The recombinant survivin of 16.5 kDa was released from the GST frame by thrombin cleavage, followed by neutralization with 1 mM benzamidine, extensive dialysis in PBS (pH 7.4), and analysis by SDS gel electrophoresis. Recombinant survivin (100 mg) was used to immunize BALB/c mice, and hybridomas were generated by the fusion of spleen cells with parental SP2/0 myeloma cells according to published protocols (13). Hybridomas were screened for reactivity with recombinant survivin by ELISA. Three positive wells were established and cloned twice by limiting dilution. One anti-survivin mAb designated 8E2 (IgG1) was selected, confirmed by ELISA and immunoblotting against the recombinant survivin, and used in the present study as culture supernatant.

Immunohistochemical Staining for Survivin and the Scoring Method for Its Expression. A pilot study using the generated anti-survivin antibody was conducted on several cases of high-grade non-Hodgkin’s lymphoma, pancreatic cancer, and gastric carcinoma to confirm the specificity in staining tumor cells and to determine an appropriate dilution for mAb 8E2 staining of gastric carcinomas. One case of stage III gastric carcinoma stained intensively and reproducibly for survivin expression in more than 30% of tumor cells, and...
Immunohistochemical staining was carried out with the standard avidin-biotin-peroxidase complex technique using the L.V. Dako LSAB kit (DAKO A/S, Carpinteria, CA) after antigen retrieval by pressure cooking. Briefly, deparaffinized sections were immersed in a 10 mm sodium citrate buffer (pH 6.0) after bringing the solution to a boil in a pressure cooker and heated two times for 3 min each at a 10-min interval while keeping the pressure indicator valve rising. After quenching in 3% hydrogen peroxide and blocking, the sections were incubated with primary antibody diluted 1:5 overnight at 4°C. Biotinylated antimouse immunoglobulin and streptavidin conjugated to horseradish peroxidase were subsequently applied. Finally, 3,3-diaminobenzidine was used for color development, and hematoxylin was used for counterstaining. Negative control slides in the absence of primary antibody were included for each staining. To quantitate the survivin expression in the various samples examined, a scoring method was modified from that for bcl-2 expression by Sinicrope et al. (14). A mean percentage of positive tumor cells was determined in at least five areas at ×400 magnification and assigned to one of the five following categories: (a) 0, <5%; (b) 1, 5–25%; (c) 2, 25–50%; (d) 3, 50–75%; and (e) 4, >75%. The intensity of survivin immunostaining was scored as follows: (a) weak, 1+; (b) moderate, 2+; and (c) intense, 3+. For tumors that showed heterogeneous staining, the predominant pattern was taken into account for scoring. The relative intensity of survivin staining defined as 3+ was identified with the intensity of bcl-2 staining in infiltrated lymphocytes. The percentage of positive tumor cells and the staining intensity were multiplied to produce a weighted score for each case. Cases with weighted scores of less than 1 were defined as negative, otherwise they were defined as positive.

Immunohistochemical Staining for bcl-2 and p53. A monoclonal mouse antibody against bcl-2 oncoprotein diluted 1:20 (clone 124; DAKO, Copenhagen, Denmark) and a mouse antihuman p53 antibody diluted 1:50 (DO-7; DAKO) were used as primary antibodies for bcl-2 and p53 immunostaining. Before the addition of the primary antibody, sections were heated in a microwave three times at 900 W for a total of 15 min. The other staining procedures were same as those for survivin. A positive control for p53 staining was obtained from previous study (15), whereas reactivity with infiltrating lymphocytes served as a positive control for bcl-2 staining. Negative controls were prepared in the absence of primary antibody as described above. The scoring criteria for bcl-2 was the same as that for survivin, and cases with weighted scores of less than 1 were judged as negative. For p53 expression, cases with less than 5% positively stained tumor cells were defined as negative, otherwise they were defined as positive.

Histochemical Detection of Apoptosis and Determination of the AI. Apoptotic cells and apoptotic bodies were visualized by in situ labeling using the ApopTag in situ detection kit (S7101-KIT; Oncor, Gaithersburg, MD) as described elsewhere (15). The AI was expressed as the ratio of positively stained tumor cells and bodies/all tumor cells; it was given as a percentage for each case and was determined according to the criteria described previously (15).

Statistical Analysis. The SPSS 6.1 J software package for Macintosh (SPSS Inc., Chicago, IL) was used for all statistical analyses. Variables associated with survivin expression as well as the correlation between survivin and p53 or bcl-2 expressions were analyzed by the $x^2$ test. Differences in the tumor cell AI for groups dichotomized according to survivin expression were checked by independent t test, and the correlation between the AI and the weighted score of survivin on a per case basis was further analyzed by Pearson's correlation coefficients test. A value of $P < 0.05$ was considered statistically significant.

Results

Generation and Characterization of Anti-Survivin mAb 8E2. Recombinant survivin expressed in BL21 E. coli as a GST fusion protein migrated with the expected $M_W$, 16,500 on SDS gel after purification and the removal of the GST frame by thrombin cleavage (Fig. 1a). Murine hybridomas were generated against recombinant survivin according to published protocols, and one of them, designated 8E2 (IgG1), was selected for further investigation. Increasing concentrations of mAb 8E2 reacted strongly with immobilized recombinant survivin by ELISA in a specific and saturable reaction, whereas control nonbinding mAb 14E11 was ineffective (Fig. 1b). Similarly, mAb 8E2 immunoblotted recombinant survivin as a single band of 16.5 kDa, whereas control mAb 14E11 did not react with survivin under the same experimental conditions (Fig. 1c).

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Fig. 1. Expression of recombinant survivin and generation of anti-survivin mAb 8E2. A, recombinant survivin was expressed as a GST fusion protein in isopropyl-1-thio-β-D-galactopyranoside-induced BL21 E. coli, isolated by affinity chromatography on a GST-Sepharose 4B column, and released from the GST frame by thrombin cleavage. Aliquots of the material at various steps during the purification procedure were analyzed by electrophoresis on a 10% SDS-polyacrylamide gel, and protein bands were visualized by Coomassie Blue staining. Mouse mAbs to purified recombinant survivin were generated according to standard hybridoma techniques and selected for reactivity with recombinant survivin. B, aliquots of recombinant, purified survivin were immobilized on plastic microtiter plates at 1 mg/ml for 1 h at 4°C. Plates were rinsed, coated with 3% gelatin, and incubated with a serial ascites dilution of the anti-survivin mAb 8E2 (B) or control mAb 14E11 (A) followed by biotin-conjugated rabbit antimouse IgG for 1 h at 37°C and streptavidin-alkaline phosphatase. Absorbance was determined an A405 using p-nitrophenyl phosphate (Sigma Chemical Co., St. Louis, MO) as a substrate. C, aliquots of recombinant survivin were separated on a 5–20% SDS-polyacrylamide gradient gel, transferred to nylon membranes, and subjected to immunoblotting with anti-survivin mAb 8E2 (Lane 1) or control mAb 14E11 (Lane 2), as described previously (6).
The Expression of Survivin and the Associated Clinicopathological Variables. Consistent with the absence of survivin transcript and protein in normal adult tissue (6), no reactivity of the anti-survivin mAb 8E2 was observed with normal gastric mucosa by immunohistochemistry. Only a faint reactivity of mAb 8E2 was occasionally observed in the foveolae. In contrast, survivin was prominently found in gastric cancer by immunohistochemistry. Positive staining for survivin was located in the cytoplasm of tumor cells, being prominent in the neoplastic glandular structure and oriented toward the luminal side in transformed cells, reminiscent of the expression of bcl-2 in these cells (7). However, unlike bcl-2, survivin expression was selectively restricted to cancer cells, and no reactivity of the anti-survivin mAb 8E2 in infiltrating lymphocytes and other stroma cells could be demonstrated (Fig. 2a). The intensity of survivin staining was usually homogeneous within a case tested, but the number of tumor cells stained positively by the anti-survivin mAb ranged between 20–100%, depending on the case examined. After multiplying the weighted survivin score, 60 cases of gastric cancer in the present series were defined as positive (34.5%), with weighted survivin scores from 1–12. A complete clinicopathological analysis of survivin-positive cases is shown in Table 1. As shown in Table 1, survivin was prominently found in tumors of the intestinal histological type without lymphatic invasion. None of the other prognostic parameters analyzed in survivin-positive cases, including different tumor stages, tumor depth, and the presence of lymph node metastasis, reached statistical significance (Table 1).

The Correlation between Survivin and p53 or bcl-2 Expression. Nuclear accumulation of p53 was demonstrated in 82 of 174 cases of gastric cancer (47.1%; Fig. 2b), without statistically significant stage differences of tumors. The expression of survivin clearly segregated with p53-positive tumors as compared with p53-negative cases [46 of 82 cases (56.1%) versus 14 of 92 cases (15.2%)]. The mean weighted survivin score of p53-positive tumors was significantly higher than that of the p53-negative tumors. The $\chi^2$ analysis confirmed a highly statistically significant correlation between survivin and p53 expression (Fig. 3a, $P < 0.001$). In parallel experiments, bcl-2 immunoreactivity was detected in only 13 of 174 cases (7.5%), with a variable proportion of tumor cells stained (5–95%). In contrast, infiltrating lymphocytes in all tumor samples prominently and uniformly stained for bcl-2 expression under the same experimental conditions and in agreement with previous observations (Fig. 2c). Coexpression of survivin and bcl-2 was observed in nine cases and had statistical significance (Table 2; $P = 0.006$).

The Relationship between Survivin Expression and Tumor Cell Apoptosis. Apoptotic cells and apoptotic bodies were detected in all cases of gastric cancer examined by in situ labeling (Fig. 2d). The mean AI was 0.85% (SD, 0.59%; range, 0.03–3.54%) with a median of 0.75%. No significant differences were observed between the AI...
that in survivin-negative tumors. Consistent with this view, expression of recombinant survivin prolonged the viability of factor-deprived pre-B lymphoma BaF3 cells (6), and other IAP proteins counteracted apoptosis induced by several stimuli in vitro (18).

Whether or not the reduced AI observed in survivin-positive tumors reflects the antiapoptosis function of survivin per se or the synergism with other inhibitors of apoptosis is currently unknown. However, the expression of bcl-2 in our series was limited to only 7.5% of the cases, in agreement with the findings of Alsabeh et al. (8) but not with the observations of Lauwers et al. (19). The sensitivity and specificity of bcl-2 immunostaining was independently confirmed and the tumor stage. The mean AI in survivin-positive tumors was 0.62% (SD, 0.39%), which was significantly lower than the mean AI of 0.97% (SD, 0.64%) observed in survivin-negative tumors (Table 2; \( P < 0.001 \)). A correlation coefficient test showed an inverse correlation between the AIs and the weighted survivin score on a per case basis \( (r = -0.23; P = 0.002; \text{Fig. 3b}) \). Finally, the AIs in bcl-2-positive tumors were significantly lower than those in bcl-2-negative tumors (Table 2; \( P = 0.008 \)).

**Discussion**

In this study, we have shown that gastric cancers expressing the novel IAP survivin (6) display a dramatically reduced AI and coexpress the tumor-regulatory molecules bcl-2 and p53. Among the recently described IAP family (16), survivin is characterized by a unique structure with a single baculovirus IAP repeat and no zinc binding domain known as RING finger (16), and by a selective distribution in common human cancers but not in normal neighboring tissue in vivo (6). Here, to gain insights into the potential mechanisms of apoptosis inhibition in gastric cancer, we have expressed survivin in E. coli as a GST fusion protein and raised a novel mAb panel against the purified recombinant molecule. The prototype mAb of this panel, mAb 8E2, stained 34.5% of stage I-III gastric carcinomas, reacting with a variable degree of intensity with 20-100% of tumor cells. In contrast, the neighboring normal tissue or the lymphoid infiltrate did not express survivin, in agreement with previous observations (6). Previous studies demonstrated that in addition to cancer cells, survivin was prominently expressed in a developmentally regulated pattern during embryonic and fetal development (17). In this context, it is possible that the presence of survivin in approximately one-third of the gastric cancer cases analyzed in the present series may reflect the different developmental phases of gene expression during malignant transformation. As one of the most significant aspects of this study, the expression of survivin in gastric cancer was significantly associated with drastically reduced apoptosis, as compared with

![Weighted Survivin Score](image1)

**Fig. 3.** a, weighted score of survivin expression on a per case basis and its correlation with p53 expression. Survivin was expressed in 15.2% (14 of 92) of p53-negative tumors and 56.1% (46 of 82) of p53-positive tumors \( (P < 0.001) \). b, correlation between tumor cell AI and weighted survivin score on a per case basis \( (r = -0.23; P = 0.002) \).

**Table 2** Correlation between tumor cell AI and expression of survivin and bcl-2

<table>
<thead>
<tr>
<th>Variable</th>
<th>Expression</th>
<th>No.</th>
<th>AI (%)</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Survivin expression</td>
<td>Negative</td>
<td>114</td>
<td>0.97 ± 0.64</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Positive</td>
<td>60</td>
<td>0.62 ± 0.39</td>
<td></td>
</tr>
<tr>
<td>bcl-2 expression</td>
<td>Negative</td>
<td>161</td>
<td>0.88 ± 0.59</td>
<td>0.008</td>
</tr>
<tr>
<td></td>
<td>Positive</td>
<td>13</td>
<td>0.43 ± 0.38</td>
<td></td>
</tr>
</tbody>
</table>
by the prominent expression of bcl-2 in infiltrating lymphocytes in all cases examined. Therefore, although the coassociation of bcl-2 and survivin in the same tumor was highly statistically significant, the decreased AI of survivin-positive cancers seems to be largely independent of bcl-2. Spontaneous apoptosis is observed much more frequently in tumor tissues than in the neighboring normal cells, which has been attributed to a lack of nutrients, competition for growth factors, or oxygen starvation due to deregulated proliferation (2, 20). However, apoptotic cell death in cancer is always lower than that potentially predicted from the growth rate of the tumor, implying a general mechanism of apoptosis inhibition in malignancies (1, 2, 15). In this context, and consistent with the paradigm of gastric cancer described here, the broad distribution of survivin in neoplasia seems to be largely independent of bcl-2. Spontaneous apoptosis is observed much more frequent

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References

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