Spontaneous Apoptosis in Advanced Esophageal Carcinoma: Its Relation to Fas Expression

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ABSTRACT

The prognostic importance of spontaneous apoptosis and its correlation with clinicopathological characteristics and Fas expression have yet to be delineated in esophageal carcinoma. Specimens from 65 patients with advanced squamous cell carcinoma of the esophagus were used for immunohistochemical evaluation of Fas, proliferating cell nuclear antigen, and apoptosis. The mean apoptotic index (AI) of 65 tumors was 1.38 ± 0.99% (range, 0.10–4.49%). Thirty-nine (60.0%) patients had a high AI, and 26 (40.0%) patients had a low AI. Low AI was correlated with advanced tumor stage ($P = 0.0197$) and weak Fas expression ($P = 0.0093$). Patients with a low AI had significantly ($P = 0.0095$) worse survival than those with a high AI. However, by multivariate analysis, low AI alone was not an independent prognosticator. When combined with cellular proliferation index, AI became an independent prognostic factor ($P = 0.0283$) in this group of patients. Our results suggest that enhanced Fas expression is responsible for high AI in squamous cell carcinoma of the esophagus. High AI, combined with the cellular proliferation labeling index, could be an independent prognostic indicator.

INTRODUCTION

Multiple factors are responsible for modulation of tumor growth and prognosis of patients with malignant tumors. Recently, much attention has been paid to the maintenance of tumor volume by cellular proliferation and apoptosis. Apoptosis is a genetically encoded program of cell death and plays a critical role in organ development and homeostasis of multicellular organisms (1). Apoptosis could be accurately identified by using the TUNEL[2] method, which targets fragmented DNA for the detection of apoptotic cells (2). Many investigators have reported that the frequency of tumor apoptosis is significantly related to prognosis in various cancers including breast (3), gastric (4), tongue (5), and colorectal carcinoma (6–8). In esophageal carcinoma, previous studies have shown that tumor apoptosis is significantly related to tumor differentiation (9) and to several apoptosis-related molecules including p53 (10) and bcl-2 (11). However, little is known about the AI, its correlation with various clinicopathological factors, and its prognostic importance.

Recently, various molecular biological factors such as the Fas/FasL system have been shown to play an important role in the regulation of tumor apoptosis. Fas (CD95) is a transmembrane protein and a member of the tumor necrosis factor receptor family. Binding of FasL to Fas induces trimerization of the Fas receptor and recruits caspase-8 via an adaptor protein called FADD/MORT1. The oligomerization of caspase-8 may result in self-activation of proteolytic activity and trigger the interleukin 1β-converting enzyme protease cascade. The activated interleukin 1β-converting enzyme members can cleave various substrates, such as poly(ADP) ribose polymerase, lamin, rho-GDI, and actin, and cause morphological changes to the cells and nuclei (12). Previously, we and others have shown that down-regulation of tumor Fas may be a hallmark of immune privilege for tumors and may also reduce Fas expression-produced poorer outcome in carcinoma patients (13–15). To date, a detailed analysis of the index of spontaneous apoptosis and its relationship to Fas expression has not been performed in human esophageal carcinomas.

An imbalance between apoptosis and proliferation is believed to underlie tumor development and prognosis. Therefore, it is important to note that a combination of measurements of proliferation and apoptosis could provide a more realistic prediction of tumor behavior. In esophageal carcinoma, the prognostic significance of tumor cell proliferation remains controversial (16, 17), and moreover, the prognostic significance of a combination of apoptosis and proliferation has not been adequately investigated. In this study, we focused on cellular apoptosis in 65 advanced esophageal SCCs, and the data were used to discover any correlation between the AI and clinicopathological factors or Fas expression.

MATERIALS AND METHODS

Surgically resected specimens were collected from 65 consecutive patients with pT2 and pT3 esophageal SCC operated on with curative intent between December 1980 and February 1998 at the Second Department of Surgery, Shimane Medical University (Shimane, Japan). None of the patients had received

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[2] The abbreviations used are: TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling; SCC, squamous cell carcinoma; PCNA, proliferating cell nuclear antigen; AI, apoptotic index; FasL, Fas ligand; PCNALI, PCNA labeling index.

preoperative radiotherapy and/or chemotherapy. The clinicopathological characteristics of these patients were investigated based on the tumor-node-metastasis (TNM) classification of esophageal SCC (18).

Detection of Tumor Apoptosis. Apoptotic cells and bodies were detected by the TUNEL method using the DeadEnd Colorimetric Apoptosis Detection System Kit (Promega, Madison, WI). A modified protocol based on the manufacturer's instructions was used. Briefly, after routine deparaffinization, rehydration, and washing in PBS, tissues were digested with proteinase K (20 μg/ml in PBS) for 30 min at room temperature and washed with PBS. Slides were then put into 0.3% H_2O_2 for 5 min and washed with PBS. After incubation with the equilibration buffer for 5 min, terminal deoxynucleotidyl transferase enzyme was pipetted onto the sections, which were then incubated at 37°C for 1 h. The reaction was terminated by stop/wash buffer. After washing the slides again with PBS, anti-digoxigenin peroxidase was added to the slides. Slides were washed, stained with diaminobenzidine, and counterstained with hematoxylin. A positive control slide was prepared by nicking DNA with DNase I (20 μg/ml) for the first staining procedure. Substitution of terminal deoxynucleotidyl transferase with distilled water was used as a negative control.

Immunohistochemical Detection of Fas and PCNA Expression. Dewaxed paraffin sections were immunostained by the streptavidin-biotin peroxidase complex method as described previously (13). Primary antibodies raised against Fas (rabbit monoclonal antibody; diluted 1:400; Wako Pure Chemical Industries, Ltd., Osaka, Japan) and PCNA (1:50 dilution for 1 h; DAKO, A/S, Denmark) were used. Aminoethylcalbasol was used as the chromogen for Fas staining, and diaminobenzidine was used for PCNA staining, and the slides were counterstained with hematoxylin. In every run, positive and negative controls were used for quality control of the staining procedure.

The intensity and extent of Fas expression were evaluated by a comprehensive score formula as described previously (13, 19). The results obtained with the two scales were multiplied against each other, yielding a single scale with scores of 1, 2, 3, 4, 6, and 9. Tumors with staining scores of ≤4 were considered to have weak Fas expression, and those with staining scores of ≥6 were considered to have strong expression.

Counting of Apoptotic and Proliferating Cells. All histological slides were examined by two observers (M. S. and S. O.) who were completely unaware of the clinical data or the disease outcome of the patients. The levels of tumor proliferation and apoptosis were expressed as a PCNALI and an AI. When evaluating the apoptotic cell number, we confirmed the presence of standard morphological characteristics of apoptosis in TUNEL-positive cells. Several high-power fields (×400) with the most abundant distribution of TUNEL-positive and PCNA-stained tumor cells were selected for counts, and between 1000 and 2000 tumor cells were counted. The AI and PCNALI were expressed as the ratio of positively stained tumor cells to all tumor cells. Bisections of the AI and PCNALI were done at several points before reaching a final cutoff value of 0.80 and 23.0 for AI and PCNALI, respectively, which represented the best predictive value for patient survival.

Statistical Analyses. The standard χ^2 test with or without Yates' correction was used for comparative analyses. The difference in the numerical data between the two groups was evaluated by using the Mann-Whitney *U* test. The correlations between parameters were evaluated by using the Spearman rank correlation test. The survival rates were estimated using the Kaplan-Meier method (20), and the statistical analysis was carried out using the log-rank test. In multivariate analysis, independent prognostic factors were determined by stepwise analysis (StatView J4.5; Abacus Concepts, Inc., Berkeley, CA). The level of significance was set at $P < 0.05$.

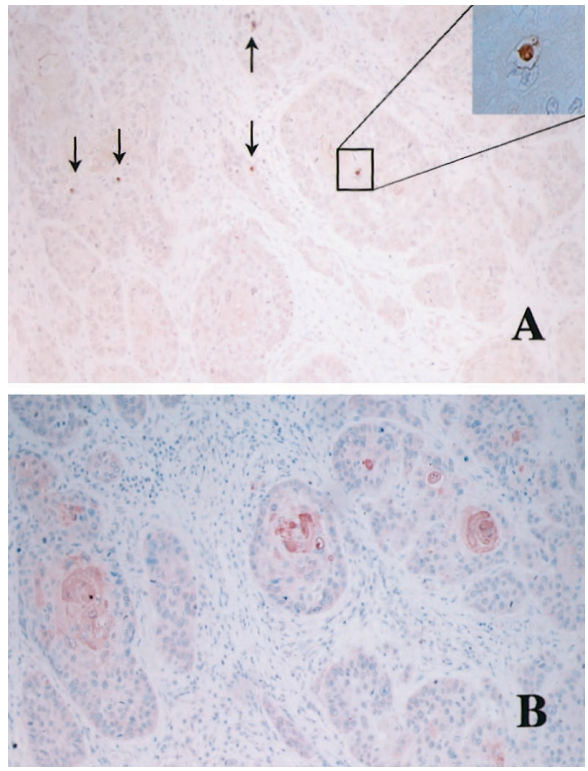

Fig. 1 Immunohistochemical staining of apoptotic tumor cells (*A*) and Fas (*B*). Fas expression was observed near the center of tumor nest in advanced tumors (*B*). TUNEL for apoptosis in a similar area of Fas expression is shown in *A*. On a TUNEL-stained section, intense TUNEL signals are visible in the nuclei of apoptotic cancer cells.

RESULTS

Patients and Tumor Characteristics. There were 58 male patients and 7 female patients. The mean age of patients was 63.7 years (age range, 46–83 years). Pathological tumor stages (pT) were pT2 in 38 patients and pT3 in 27 patients.

Cell Apoptosis in Tumors. Apoptotic tumor cells were clearly identified by brown nuclear staining using the TUNEL method (Fig. 1*A*). Positive staining for apoptosis was detected in all tumors. The mean AI of 65 tumors was 1.38 ± 0.99% (range, 0.10–4.49%). Thirty-nine patients (60.0%) had a high AI, whereas 26 (40.0%) patients had a low AI. Correlations between AI and clinicopathological factors are shown in Table 1. There was no relationship between AI and the depth of tumor invasion (T) and lymph node metastasis (N); however, low AI was correlated with advanced tumor stage ($P = 0.0197$).

Table 1 Relationship between AI and clinicopathological and biological features in advanced esophageal carcinomas ($n = 65$)

Variable	High AI ($n = 39$)	Low AI ($n = 26$)
Age (yrs)	63.46 ± 10.09	64.08 ± 7.65
Gender (male:female)	34/5	24/2
Tumor size (cm)	4.95 ± 1.75	5.37 ± 1.67
Histological differentiation (well/others)	18/21	8/18
pT (pT2/pT3)	26/13	12/14
pN (positive/negative)	23/16	20/6
TNM stage (≤II/≥III)	23/16[a]	8/18
Fas (strong/weak)	20/19[b]	5/21
PCNALI	32.05 ± 16.60	32.54 ± 20.31

[a] $P = 0.0197$ (Mann-Whitney U test).
[b] $P = 0.0093$ (χ^2 test).

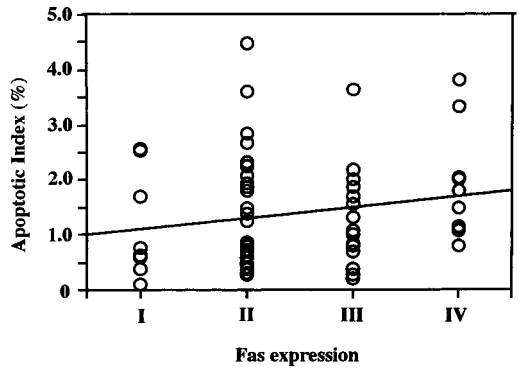

Fig. 2 We semiquantitatively scored four groups for Fas expression: (*a*) group I, Fas expression score of 1; (*b*) group II, Fas expression score of 2, 3, or 4; (*c*) group III, Fas expression score of 6; and (*d*) group IV, Fas expression score of 9. A significant correlation between the AI and Fas expression ($\rho = 0.278$; $P = 0.026$) was observed by using the Spearman rank correlation coefficient. The number of patients in the four groups was as follows: (*a*) group I, 8 patients; (*b*) group II, 32 patients; (*c*) gropu III, 15 patients; and (*d*) group IV, 10 patients.

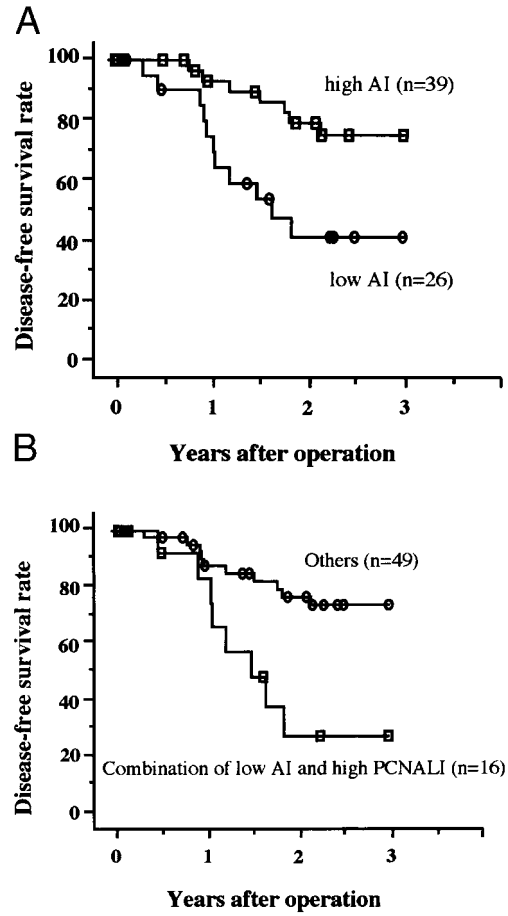

Fig. 3 Disease-free survival curves of patients with high AI and low AI (*A*, $P = 0.0095$, log-rank test). Disease-free survival curves of patients with a combination of low AI and high PCNALI and others (*B*, $P = 0.0031$, log-rank test).

Fas Expression. Fas expression was predominant near the center of tumor nests (Fig. 1*B*). Strong Fas expression was detected in 25 of 65 (38.5%) tumors. High AI was more frequent in the group with strong Fas expression ($P = 0.0093$; Table 1). According to the Spearman correlation coefficient, a significant correlation between the tumor AI and Fas expression was observed ($\rho = 0.278$; $P = 0.026$; Fig. 2).

Cell Proliferation in Tumors. Proliferating tumor cells were clearly identified by brown nuclear staining by PCNA immunohistochemistry. The PCNALI of advanced esophageal carcinoma in our series ranged from 0.50–73.65% (mean, 32.25 ± 18.02%). Forty patients (61.5%) had a high PCNALI, whereas 25 patients (38.5%) demonstrated low PCNALI. No significant correlation could be found between PCNALI and AI in this series of patients (Table 1).

Long-term Survival. At the time of this analysis, 25 patients were alive and cancer free, 22 patients had died of causes unrelated to esophageal cancer, and the remaining 18 patients had died of recurrent disease.

The 3-year survival rates of patients with high and low AI tumors were 76.9% and 45.6%, respectively. The patients with low AI had poorer outcomes for disease-free survival ($P = 0.0095$; Fig. 3*A*). Also, the combination of AI and PCNALI revealed that cases with low AI and high PCNALI had a significantly worse survival by univariate analysis ($P = 0.0031$; Fig. 3*B*). PCNALI did not influence the survival rate ($P = 0.4069$). In multivariate analysis, AI alone was not detected as an independent prognostic factor for disease recurrence ($P = 0.0905$; Table 2, model I). However, AI, when coupled with PCNALI, became an independent prognosticator by multivariate analysis ($P = 0.0283$; Table 2, model II). With regard to tumor Fas expression, patients with low Fas expression had poorer outcomes by univariate analysis ($P = 0.0093$). However, due to the existence of a strong correlation between AI and Fas expression, Fas expression was excluded from multivariate analysis.

DISCUSSION

SCC of the esophagus is one of the most malignant tumors, and patients with this disease have a dismal prognosis. Due to early diagnosis and effective radiochemotherapy, the survival

Table 2 Multivariate analysis of possible prognostic factors for survival

Variable	Category	*P*	
		Model I	Model II
Age (yrs)	<60, ≥60	0.2062	0.5207
Gender	Male, female	0.4678	0.5634
Tumor size (cm)	<5, ≥5	0.1554	0.5120
Histology	Well, others	0.7013	0.6095
PT status	2, 3	0.8380	0.7610
PN status	0, 1	0.0110	0.0170
AI	High, low	0.0905	
PCNA LI	High, low	0.5146	
Combination of AI and PCNALI	Low AI and high PCNALI, others		0.0283

rate of patients has improved recently (21, 22). The most significant clinicopathological indicator of survival in the patients with esophageal carcinoma is the presence or absence of regional lymph node metastasis (23–25). Recently, intensive molecular and biological studies have demonstrated the significance of oncogenes, tumor suppressor genes, and growth factors in carcinogenesis and malignant transformation of cells.

Apoptosis might be regulated by various factors such as tumor necrosis factor α, FasL, tumor necrosis factor-related apoptosis-inducing ligand, and various oncogenes, including *p53, bcl-2, c-myc, ras,* and *c-fos*. We have demonstrated previously that the Fas/FasL system significantly affects the survival of esophageal carcinoma patients, with decreased survival seen in patients with Fas down-regulation (13). Likewise, recent studies in lung (15) and hepatocellular carcinomas (14) have reported that weak Fas expression is correlated with poor prognosis. The Fas/FasL system mediates T-cell cytotoxicity, and, thus, FasL-positive T cells might eliminate Fas-positive tumor cells by inducing apoptosis. The loss of Fas expression might result in reduced sensitivity of the tumor cells to the cytotoxic activity of T lymphocytes (26). However, it is unclear whether tumor apoptosis actually increases in patients with strong Fas expression in esophageal carcinoma. In the present study, we detected a positive correlation between Fas expression and apoptosis. This finding is in agreement with the report of Nagao *et al.* (14) on hepatocellular carcinoma. Bennett *et al.* (27) have shown that SCC of the esophagus expresses FasL. Hence, decreased expression of Fas could impair apoptosis of esophageal tumor cells not only in response to antitumor T cells, but also in response to autocrine suicide via tumor-expressed FasL. This could account in part for the lower AI in tumors with low Fas expression. Therefore, it could be suggested that Fas expression plays a key role in apoptosis in esophageal SCC and may help in designing new therapeutic approaches based on reinforcement of Fas/FasL-induced tumor apoptosis.

As shown by the results of the present study, low AI was significantly correlated with advanced tumor stage in esophageal carcinoma. Our results are similar to those of previous studies that showed that low AI is correlated with progression of tumor depth and positive lymph node metastasis in colon (8) and esophageal carcinoma (28). It appears that a low rate of tumor apoptosis is correlated with tumor progression. Moreover, apoptosis occurred more frequently in well-differentiated tumors than in poorly differentiated tumors in colon (6, 8), tongue (5), and gastric cancer (29), indicating that apoptosis occurred more frequently in slow-growing tumors than in rapidly growing tumors. Also, in esophageal carcinoma, Ohbu *et al.* (28) reported that the rate of apoptosis was higher in well-differentiated tumors than in poorly differentiated tumors. These results are in agreement with the present study. Therefore, esophageal cancer patients with a low AI showed a poor outcome compared with those with a high AI.

It has been shown that proliferation plays an important role in tumor progression; however, net tumor growth is not regulated by tumor proliferation alone, and the balance of cell apoptosis and proliferation is important (30). Thus, in a clinical situation, simultaneous evaluation of both apoptosis and proliferation might be useful for predicting tumor progression and patient survival. In the present study, spontaneous apoptosis alone did not become an independent prognosticator, but the combination of apoptosis and proliferation did. To our knowledge, this is the first report demonstrating a prognostic significance of combined apoptosis and cellular proliferation in esophageal carcinoma. Similarly, in patients with adenocarcinoma of the cervix, AI had no impact on patient survival (31). However, when the mitotic index was assessed along with the AI, the ratio of AI:mitotic index had a significant impact on patient survival (31). In colon carcinoma, patients with low apoptosis and high proliferation more frequently had advanced Dukes' stage tumors and lymph node metastasis (8). In this study, the combination of apoptosis and proliferation was also correlated with large tumor size ($P = 0.04$), indicating that net tumor growth represented by a combination of apoptosis and proliferation would be a more appropriate tool to assess tumor aggressiveness and patient survival in esophageal SCC.

In conclusion, enhanced Fas expression is responsible for high AI, and high AI could be an independent prognostic indicator when coupled with the concomitant cellular proliferation labeling index in esophageal SCC.

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