Up-Regulation of Fas (APO-1/CD95) Ligand and Down-Regulation of Fas Expression in Human Esophageal Cancer

Catherine Gratas, Yasuo Tohma, Christoph Barnas, Philippe Tanière, Pierre Hainaut, and Hiroko Ohgaki

Abstract

Fas (APO-1/CD95) is a cell surface receptor that mediates apoptosis when it reacts with Fas ligand (FasL) or Fas antibody. In this study, we analyzed Fas and Fasl expression in normal esophageal mucosa and esophageal squamous cell carcinomas. Reverse transcriptase-PCR revealed that Fas, soluble Fas, and Fasl were expressed in all eight esophageal squamous carcinoma cell lines analyzed. Furthermore, it was demonstrated that Fasl expressed in esophageal carcinoma cells is functional because coculture experiments using Fasl-expressing TE-15 esophageal carcinoma cells resulted in apoptosis of Jurkat T leukemia cells, which are sensitive to Fas-mediated apoptosis. Immunohistochemistry of Fas and Fasl showed that they are constitutively expressed in normal esophageal mucosa, Fasl being predominantly in the basal and suprabasal layers, whereas Fas is in more differentiated layers, i.e., rows of polyhedral cells of the intermediate layers and squamous cells forming the outer layers. In 18 of 19 invasive esophageal squamous cell carcinomas, Fasl expression was found in >50% of tumor cells. In contrast, most tumors (15 of 19, 79%) either showed no Fas expression or showed expression in <5% of tumor cells. These alterations were already detected in dysplasia and carcinoma in situ. These results suggest that up-regulation of Fasl and down-regulation of Fas expression are early and frequent events associated with the evolution of esophageal squamous cell carcinomas.

Introduction

Esophageal cancer is one of the 10 most frequent types of cancer worldwide, particularly in developing countries. It is also one of the most lethal cancers, with a 5-year survival below 10% (1). Its occurrence is clearly associated with exogenous risk factors that include alcohol consumption and tobacco smoking in Western countries and Japan and a variety of dietary habits or vitamin and oligo-element deficiencies in several regions of China, South America, southeast Asia, and Africa (2).

The evolution of squamous cell carcinoma of the esophagus is associated with multiple genetic alterations, including loss of heteryozygosity on chromosomes 3p, 5p (APC locus), 9p, 9q, 13q (Rb locus), 17p (p53 locus), and 18q (DCC locus), as well as amplification of the epidermal growth factor receptor, HER-2, c-myc, and cyclin D1 genes [see review, see Montesano et al. (3)]. The most frequent genetic alteration in squamous cell carcinoma of the esophagus is point mutation of the p53 gene (40-60%), which occurs at a relatively early stage of tumor development. Mutations of the p16 (MTS-1) gene have also been detected in 10-20% of the cases [for review, see Montesano et al. (3)]. Although the temporal sequence of these events is far from being understood, these observations point to an essential role of alterations in G1-S regulatory genes. However, little is known about alterations of genes involved in the control of apoptosis in esophageal cancer, except for reports showing that bcl-2 protein, an inhibitor of apoptosis, is overexpressed in 32-58% of esophageal squamous cell carcinomas (4, 5). We, therefore, studied the expression of Fasl3 and Fas receptor (APO-1/CD95), which are involved in one of the major apoptotic pathways, in normal esophagus and in esophageal squamous cell carcinomas.

FasL belongs to the tumor necrosis factor superfamily of membrane and secreted proteins (6), and Fas is a member of the respective receptor family of cell surface proteins (6, 7). The Fas/FasL system is involved in the induction of apoptosis in activated T cells, B cells, and natural killer cells (8-11). In addition to the immune system, Fas is expressed in some adult tissues, including liver, ovary, thymus, heart, and skin (12-14). FasL expression was first considered to be restricted to the immune system, including activated T cells (9), B cells (10), and natural killer cells (11). However, the identification of Fasl expression by cells in immune-privileged sites, such as stroma cells of the eye and Sertoli cells of the testis, suggested that Fasl may be important for maintaining immune privilege [see review by Nagata (6)]. Furthermore, constitutive expression of Fas and Fasl has been observed in a variety of adult mouse tissues that are characterized by high rates of apoptotic cell death, suggesting that the Fas/Fasl system may also be implicated in apoptotic cell death during physiological cell turnover (14). More recently, induction or up-regulation of functional Fasl expression has been detected in several human neoplasms (15-21).

In this study, we show the first evidence that Fasl and Fas are constitutively expressed in normal human esophageal mucosa, Fasl being found predominantly in the basal and suprabasal layers and Fas being found in more differentiated layers, and that up-regulation of Fasl and down-regulation of Fas expression are frequent in esophageal squamous cell carcinomas.

Materials and Methods

Primary Esophageal Cancer and Esophageal Carcinoma Cell Lines.

Four primary esophageal squamous cell carcinomas were obtained during surgical procedures at the Faculty of Medicine, Prince of Songkla University (Songkla, Thailand). They were frozen in liquid nitrogen and kept at −80°C until RNA extraction.

Nineteen primary invasive esophageal squamous cell carcinomas were collected during surgical procedures at University Hospital (Zürich, Switzerland) and at Edouard Herriot Hospital (Lyon, France) and were fixed in buffered formalin and embedded in paraffin. Tumors were classified into well, moderately, or poorly differentiated squamous cell carcinomas according to
cells were cocultured with TE-15 esophageal cancer cells at a ratio of 1:20 for 24, 48, and 72 h. As a control, TE-11 cell line was from poorly differentiated squamous cell carcinomas, and TE-9 and TE-13 cell lines were from well-differentiated squamous cell carcinomas. TE-15 cell lines were derived from moderately differentiated squamous cell carcinomas (23).

Fas and FasL expression in primary esophageal squamous cell carcinomas

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* Cells with FasL or Fas expression were recorded as follows: -, no expression; +, <5% positive cells; ++, 5-25% positive cells; ++++, 26-50% positive cells; +++++, >50% positive cells.

** With keratinization.

WHO classification (22). The age and sex of patients and differentiat ion status of squamous cell carcinomas are shown in Table 1.

Esophageal squamous cell carcinoma cell lines TE-1, TE-3, TE-6, TE-9, TE-10, TE-11, TE-13, and TE-15, which were established by Nishihira et al. (23), were cultured in RPMI 1640 supplemented with 10% FCS, penicillin, and streptomycin/glutamine, as described previously (24). TE-1, TE-3, TE-6, TE-10, and TE-15 cell lines were derived from well-differentiated squamous cell carcinomas, TE-11 cell line was from moderately differentiated squamous cell carcinomas, and TE-9 and TE-13 cell lines were from poorly differentiated squamous cell carcinomas (23).

RT-PCR for Fas and FasL. Polyadenylated RNA was extracted from the four primary esophageal tumors using the MicroQuickPrep mRNA purification kit (Pharmacia, Uppsala, Sweden), and total RNA was extracted from esophageal tumor cell lines using the Trizol reagent (Life Technologies, Inc., Gaithersburg, MD), according to the manufacturer's specifications. cDNA was synthesized using Superscript II (Life Technologies, Inc.) and oligo(dT) priming (Pharmacia), as recommended in the protocol provided. PCR then carried out for Fas transmembrane domain and FasL, as described previously (19). The primers used for amplification generated products of 344 bp for FasL and 150 bp for Fas (20, 25). All primer sets were designed to amplify sequences spanning introns, to eliminate possible false-positive amplifications due to DNA contamination. As a positive control, B-actin fragment was amplified as described previously (25).

The specificity of the PCR products was verified by Southern hybridization, as described previously (19). The DNAs used as probes were purified PCR products that cover the FasL cDNA between nucleic acids 350 and 694 (19), the Fas cDNA between nucleic acids 664 and 814, and soluble Fas cDNA between nucleic acids 664 and 814 Δ (700-762; Ref. 25).

Apoptosis Assay. Fas-sensitive Jurkat T leukemia cells, which express Fas and are sensitive to FasL-induced apoptosis, were obtained from Dr. S. Chow, University of Leicester, Medical Research Council (Leicester, United Kingdom). Fas-resistant Jurkat cells, subclones derived from Jurkat T leukemia cells that revealed a reduced sensitivity to Fas-induced apoptosis, were a kind gift from Dr. E. Goiliot, Laboratoire de Biologie Moléculaire et Cellulaire, Ecole Normale Supérieure (Lyon, France). Fas-sensitive and -resistant Jurkat T leukemia cells were maintained in RPMI 1640 supplemented with 10% FCS, penicillin, and streptomycin/glutamine. Fas-sensitive Jurkat T leukemia cells (floating in the medium) were cocultured with TE-15 esophageal cancer cells (attached to dishes) at a ratio of 1:20 for 24, 48, and 72 h. As a control, Fas-sensitive Jurkat T leukemia cells were cultured in TE-15 cell-conditioned medium for 72 h. As another negative control, Fas-resistant Jurkat T leukemia cells were cocultured with TE-15 esophageal cancer cells at a ratio of 1:20 for 72 h. After coculture, Fas-sensitive and -resistant Jurkat T leukemia cells were collected and analyzed for DNA content by flow cytometry. Fas-sensitive Jurkat T leukemia cells were also analyzed by genomic DNA fragmentation by in situ apoptosis assay (TUNEL assay). Experiments at 72 h were performed in duplicate.

For flow cytometry analysis, Jurkat T leukemia cells were washed with PBS and labeled with PI using the Cycle Test Plus DNA Reagent Kit (Becton Dickinson), according to the manufacturer's instructions. The DNA content of 20,000 PI-labeled Jurkat cells was analyzed using a FACScalibur flow cytometer (Becton Dickinson), according to the manufacturer's instructions. The percentages of G1, S, G2, and apoptotic cells were determined using the CellQuest Software cytometer (Becton Dickinson), according to the manufacturer's instructions.

TUNEL assay was carried out using Genzyme TACS in situ apoptosis detection kit (Genzyme, Cambridge, MA), according to the manufacturer's instructions. Briefly, Jurkat T leukemia cells, with and without coculture with TE-15 cells for 72 h, were fixed in 10% formalin solution for 10 min, incubated for 10 min in 80% ethanol at room temperature, and pipetted onto the slides. After protease K (10 mg/ml; Boehringer Mannheim, Lewes, United Kingdom) digestion at room temperature for 5 min, cells were incubated with Klenow labeling reaction solution in a humidified chamber at 37°C for 1 h. Cells were washed in stop buffer for 5 min, then incubated in TBL streptavidin-horseradish peroxidase detection solution for 10 min at room temperature. The reaction products were visualized with TACS blue label and counterstained weakly with eosin. In each specimen, 300-400 cells were counted using a grid under ×400 magnification. The positive nuclei that were stained with blue and exhibited morphological changes suggestive of apoptosis were counted, and the apoptotic index (%) was determined.

Immunohistochemistry for FasL and Fas. The sections were deparaffinized in xylene, rehydrated in graded ethanol, and incubated in 0.3% H2O2 solution in methanol for 20 min to block the endogenous peroxidase. The sections were then washed with water for 5 min and with PBS for 5 min. The sections for FasL immunohistochemistry were boiled in 10 mM sodium citrate (pH 6.0) for 10 min in a pressure cooker and allowed to cool at room temperature. After incubation in 5% skimmed milk for 2 h at room temperature, the sections were incubated overnight at 4°C with polyclonal antihuman FasL antibody (Q20, diluted 1:200; Santa Cruz Biotechnology, Santa Cruz, CA) or with polyclonal antihuman Fas antibody, which recognizes Fas extracellular domain (kindly supplied by Dr. Nakanishi, Kanazawa University, Kanazawa, Japan), diluted 1:1000. The reaction was visualized using Vectastain Elite ABC kit and a 3,3'-diaminobenzidine solution (Vector Laboratories, Burlingame, CA). Sections were then slightly counterstained with hematoxylin. Formalin-fixed, paraffin-embedded sections of mouse testis, thymus, and spleen were used as positive controls for FasL, and sections of normal human liver served as positive controls for Fas. Sections without primary antibodies as well as those with nonimmunized rabbit serum served as negative controls.

Carcinoma cells with FasL or Fas expression were recorded as follows: -, no expression; +, <5% positive cells; ++, 5-25% positive cells; ++++, 26-50% positive cells; +++++, >50% positive cells. Normal esophageal mucosa, dysplasia, and carcinoma in situ, which were observed adjacent to invasive squamous cell carcinomas in some sections, were also carefully examined.

Results

RT-PCR for FasL and Fas in Primary Esophageal Cancer and in Esophageal Carcinoma Cell Lines. In the four primary esophageal squamous cell carcinomas analyzed, FasL expression was detected in all four tumors, whereas Fas and soluble Fas were expressed in two tumors by RT-PCR.

RT-PCR revealed that FasL and Fas were expressed in all eight esophageal squamous cell carcinoma cell lines analyzed (Fig. 1). In all eight cases, a shorter PCR product (87 bp) was obtained together with the 150-bp PCR product from normal Fas (Fig. 1), suggesting the presence of soluble Fas (26, 27). The specificity of the PCR products was confirmed by hybridization to FasL (Fig. 1) and to Fas probes (data not shown).
Jurkat cells, which were incubated in TE-15-conditioned medium for 72 h (c and f).

The proportion of apoptotic cells increased in a time-dependent manner. After coculture for 72 h, apoptotic indices in Fas-sensitive Jurkat cells were 40% in flow cytometry analyses and 42% in TUNEL assay (Fig. 2). A fraction of apoptotic cells were detected after 24 h of coculture (12%), and the proportion of apoptotic cells increased in a time-dependent manner. After coculture for 72 h, apoptotic indices in Fas-sensitive Jurkat cells were 40% in flow cytometry analyses and 42% in TUNEL assay (Fig. 2). These values were 20 times higher than those observed in Fas-sensitive Jurkat cells cultured in TE-15-conditioned medium for 72 h (2% detected with both flow cytometry and TUNEL assay; Fig. 2, c and e). In contrast, the apoptotic fraction of Fas-resistant Jurkat cells after 72 h of incubation with TE-15 cells was 22% (Fig. 2b), only twice as much as that observed in Fas-resistant Jurkat cells, which were incubated in TE-15-conditioned medium for 72 h (11%; Fig. 2a). No indication of induction of apoptosis in TE-15 cells was observed (data not shown).

Apoptosis Assay. Fas-sensitive Jurkat T leukemia cells were cocultured with TE-15 cells, and the proportion of apoptotic Jurkat cells was determined by flow cytometry (fraction of cells with sub-G1 content) and by in situ apoptosis (TUNEL) assay (Fig. 2, c-f). A fraction of apoptotic cells were detected after 24 h of coculture (12%), and the proportion of apoptotic cells increased in a time-dependent manner. After coculture for 72 h, apoptotic indices in Fas-sensitive Jurkat cells were 40% in flow cytometry analyses and 42% in TUNEL assay (Fig. 2, d and f). These values were 20 times higher than those observed in Fas-sensitive Jurkat cells cultured in TE-15-conditioned medium for 72 h (2% detected with both flow cytometry and TUNEL assay; Fig. 2, c and e). In contrast, the apoptotic fraction of Fas-resistant Jurkat cells after 72 h of incubation with TE-15 cells was 22% (Fig. 2b), only twice as much as that observed in Fas-resistant Jurkat cells, which were incubated in TE-15-conditioned medium for 72 h (11%; Fig. 2a). No indication of induction of apoptosis in TE-15 cells was observed (data not shown).

Immunohistochemistry for FasL and Fas. Immunohistochemically, both FasL and Fas were found to be constitutively expressed in normal esophageal mucosa, with FasL being predominant in the basal and suprabasal layers and Fas being predominant in more differentiated layers, i.e., rows of polyhedral cells of the intermediate layers and squamous cells forming the outer layers of normal esophageal mucosa (Fig. 3a).

FasL protein was detected in all 19 primary esophageal squamous cell carcinomas analyzed (Table 1). Except for one tumor (case 17), FasL expression was clearly observed in the cytoplasmic membrane and cytoplasm of >50% of tumor cells (Table 1 and Fig. 3, c and d). The level of FasL expression was either similar to or higher than that in the basal layer of normal esophageal mucosa.

In contrast, most tumors (15 of 19, 79%) showed complete loss of Fas expression or expression in <5% of tumor cells (Table 1 and Fig. 3, c and d). Most Fas-positive cells were differentiated, relatively large squamous neoplastic cells located near the center of tumor nests in well-differentiated squamous cell carcinomas (Table 1 and Fig. 3d), but there was no correlation between keratinization and Fas expression (Table 1). An increase in FasL expression with low or loss of Fas expression was already observed in all precursor lesions analyzed (two dysplasias and two carcinomas in situ; Fig. 3b).

Positive controls, i.e., human normal liver for Fas immunohistochemistry and mouse testis, thymus, and spleen for FasL immunohistochemistry, were consistently positive, whereas negative controls, i.e., sections without primary antibody and those with nonimmunized rabbit serum for Fas and FasL immunohistochemistry, were consistently negative.

Discussion

Here, we show that both FasL and Fas are constitutively expressed in normal esophageal mucosa. FasL expression was predominant in the basal and suprabasal layers, whereas Fas expression was in more differentiated layers, i.e., rows of polyhedral cells of the intermediate layers and squamous cells forming the outer layers. Thus, Fas and FasL expression appear to correlate with the differentiation status of squamous epithelium, and they are not coexpressed at significant amounts in the same cells in normal esophageal mucosa. The biological significance of FasL expression in basal cells of the normal esophagus remains to be investigated. One possibility could be that FasL expression contributes to the protection of basal cells from apoptotic cell death by lymphocytes,

![Fig. 2. Flow cytometry analyses (c and d) and TUNEL assays (e and f) for Fas-sensitive Jurkat T leukemia cells incubated with TE-15 cells for 72 h (d and f) and, as a control, those cultured in TE-15-conditioned medium for 72 h (c and e). Flow cytometry analysis showed an increase in apoptotic cell fractions with hypodiploid DNA content (d, 40%) compared to control (c, 2%). TUNEL assay showed apoptotic cells (blue staining) in Jurkat T leukemia cells after coculture with TE-15 cells (f) but not in control cells (e). In the experiment using Fas-resistant Jurkat cells, only a slight increase in apoptotic cell fraction upon coculture with TE-15 esophageal cancer cells (b, 22%) was observed compared to control cells incubated in TE-15-conditioned medium (a, 11%). Magnification (e and f), ×500.](cancerres.aacrjournals.org)

![Fig. 3. c and d). Most Fas-positive cells were differentiated, relatively large squamous neoplastic cells located near the center of tumor nests in well-differentiated squamous cell carcinomas (Table 1 and Fig. 3d), but there was no correlation between keratinization and Fas expression (Table 1). An increase in FasL expression with low or loss of Fas expression was already observed in all precursor lesions analyzed (two dysplasias and two carcinomas in situ; Fig. 3b). Positive controls, i.e., human normal liver for Fas immunohistochemistry and mouse testis, thymus, and spleen for FasL immunohistochemistry, were consistently positive, whereas negative controls, i.e., sections without primary antibody and those with nonimmunized rabbit serum for Fas and FasL immunohistochemistry, were consistently negative.

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Fig. 3. Immunohistochemical analysis of FasL (left) and Fas (right) in normal esophageal mucosa (a), dysplasia (b), and invasive esophageal squamous cell carcinoma (c and d). a, FasL expression was predominant in the basal and suprabasal layers, whereas Fas expression was in rows of polygonal cells of the intermediate layers and squamous cells forming the outer layers of normal esophageal mucosa. b, dysplastic squamous cells showed loss of Fas expression and strong expression of FasL. c, invasive esophageal squamous cell carcinoma showed loss of Fas and strong expression of FasL. d, most Fas-positive cells, if present, were differentiated large squamous neoplastic cells located near the center of tumor nests. Magnifications, ×300 (a) and ×150 (b–d).
which are present in normal esophageal mucosa (28). In relation to this hypothesis, it has also been reported that basal cells of normal esophageal mucosa but not cells located in more differentiated layers express bcl-2 protein (4), suggesting that basal cells in the esophagus may possess several molecular defenses against apoptotic signals.

This study clearly shows that up-regulation of FasL is frequent in esophageal squamous cell carcinomas. It was also demonstrated that FasL expressed in esophageal carcinoma cells is functional because coculture experiments using FasL-expressing TE-15 esophageal carcinoma cells resulted in induction of apoptosis of Fas-sensitive Jurkat T leukemia cells. In contrast, induction of apoptosis in Fas-resistant Jurkat T leukemia cells upon coculture with TE-15 esophageal cancer cells was significantly less. Furthermore, there was no evidence of induction of apoptosis in Fas-sensitive Jurkat T leukemia cells after incubation in TE-15-conditioned medium. These results strongly suggest that FasL expressed in TE-15 cells are functional and that induction of apoptosis in Fas-sensitive Jurkat T leukemia cells was due to direct interaction between Fas and FasL. Induction of up-regulation of functional FasL expression has been reported in hepatocellular carcinomas (15), melanomas (16), lung carcinomas (17), glioblastomas (18, 19), a colon carcinoma cell line (20), and liver metastases of colon adenocarcinomas (21). Hahne et al. (16) reported that injection in wild-type mice of mouse melanoma cells expressing FasL led to rapid tumor formation, whereas tumorigenesis was delayed in Fas-deficient lpr mutant mice, in which immune effector cells cannot be killed by FasL. Thus, attack of Fas-expressing cytotoxic T cells by up-regulated FasL appears to be a common strategy for neoplastic cells to escape immune rejection.

Fas expression was completely lost or limited to only a minor population of neoplastic cells in the majority of esophageal squamous cell carcinomas in this study. Down-regulation of Fas expression may be a mechanism for esophageal squamous carcinoma cells to escape attack by activated cytotoxic T cells, which are frequently present in these tumors (29). Similar findings were reported by Strand et al. (15), who showed significant loss of Fas expression in the majority of hepatocellular carcinomas, whereas normal hepatocytes constitutively express Fas.

RT-PCR in this study revealed that soluble Fas was expressed in all primary esophageal tumors and esophageal tumor cell lines that expressed Fas. Soluble Fas, which is generated by alternative mRNA splicing (26, 30), has been detected in the supernatants of activated human lymphocytes (26, 30) and several human primary tumors and tumor cell lines, including glioblastomas (18, 19, 25), hepatomas (31), osteosarcomas (32), leukemia and lymphomas (27, 30) and sera from cancer patients with a variety of solid tumors or lymphomas/leukemias (27, 33). Soluble Fas antagonize cell surface Fas function (34), and thus, tumor cells with reduced normal Fas expression and with soluble Fas expression may enhance their resistance to apoptosis by FasL-expressing activated T cells.

In this study, four carcinomas with >5% Fas-positive cells were all well-differentiated squamous cell carcinomas (Table 1), and most Fas-positive cells were differentiated large squamous neoplastic cells located near the center of tumor nests (Fig. 3d). This suggests that Fas expression is associated with the status of squamous cell differentiation in carcinoma cells, as observed in normal esophageal mucosa. Ohbu et al. (35) have shown that the apoptotic labeling index detected by the TUNEL method was 18.5% in well-differentiated squamous cell carcinomas, much higher than that in poorly differentiated squamous cell carcinomas (6.8%). Whether a higher apoptotic index is due to more frequent Fas expression in well-differentiated than in poorly differentiated squamous cell carcinomas remains to be clarified.

Invasive squamous cell carcinomas of the esophagus are considered to be generally derived from dysplasia or carcinoma in situ (22). Here, we show that up-regulation of FasL and down-regulation of Fas are already observed in all lesions of dysplasia and carcinoma in situ analyzed, suggesting that they may be early alterations in the evolution of esophageal squamous cell carcinomas.

In summary, constitutive expression of Fas and FasL appears to correlate with the differentiation status of normal esophageal mucosa. The majority of invasive esophageal squamous cell carcinomas show up-regulation of FasL, which may lead tumor cells to counteract the immune system by killing Fas-expressing cytotoxic T cells. At the same time, a majority of invasive esophageal squamous cell carcinomas show down-regulation of Fas, suggesting that this may be a common mechanism for esophageal carcinoma cells to evade Fas-mediated killing. Up-regulation of FasL and down-regulation of Fas are already detected in dysplasia and carcinoma in situ, suggesting that they are early events. Furthermore, frequent soluble Fas expression may also contribute to resistance to apoptosis induced by FasL-expressing activated T cells in esophageal squamous cell carcinomas.

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