RESEARCH ARTICLE

Effects of FasL Expression in Oral Squamous Cell Cancer

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Abstract

Purpose: To probe the role of FasL in cell apoptosis in oral squamous cell carcinomas (OSCCs). Methods: The expression of Fas/FasL was assessed in 10 cases of normal oral epithelium, 38 cases of OSCC and tumor infiltrating lymphocytes (TIL), and 11 cases of metastatic lymph nodes by immunohistochemistry. Apoptosis of tumor cells and TIL was detected by terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling assay (TUNEL). FasL-induction of T cell apoptosis was tested by co-culture assay *in vitro* with SCC-9 and Jurkat T cells. Results: The 10 cases of normal oral epithelium all demonstrated extensive expression of Fas, the positive rate being largely down-regulated in OSCC (21/38) (P<0.05) compared to the normal (10/10). At the same time, the positive rate of FasL significantly increased in OSCC (P<0.05) especially those with lymph node metastasis (P<0.05). The positive rates of Fas in well and middle differentiated OSCC were higher than those in poor differentiated OSCC (P<0.05). The AI of tumor cells in Fas-positive OSCC was remarkably higher than that in Fas-negative OSCC (P<0.01), with a positive correlation between Fas expression and cell differentiation as well as apoptosis (r=0.68, P<0.01). The AI of tumor cells in FasL positive OSCC was remarkably lower than that in control while the AI of TIL was higher than in FasL negative OSCC (P< 0.05). The AI of tumor cells reversely correlated with that of TIL (r = -0. 72, P<0.05). It was found that SCC-9 cells expressing functional FasL could induce apoptosis of Jurkat cells as demonstrated by co-culture assays. As a conclusion, it is evident that OSCC cells expressing FasL can induce apoptosis in Fas-expressing T cells. Conclusions: In progression of OSCC, expression of the Fas/FasL changes significantly. The results suggest that FasL is a mediator of immune privilege in OSCC and may serve as an marker for predicting malignant change in oral tissues.

Keywords: Oral SCC - lymphatic metastasis - FasL - Fas - apoptosis

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Introduction

Malignant tumors of the oral cavity account for approximately 30% of all head and neck cancers, and 80% of these tumors are oral squamous cell carcinomas (OSCC). OSCC is one of the most common malignancies affecting people in China, and its incidence is rising (de Camargo Cancela et al., 2010; Weinberg et al., 2002). Tumor immune evasion has been shown to promote the progression of many solid tumors. Tumors use multiple mechanisms for escape, including defective antigen presentation, interference with tumor-T cell interaction, and production of immunosuppressive factors. Another possible escape mechanism is Fas-mediated T cell apoptosis (Töpfer et al., 2011).

The Fas ligand (FasL) and its receptor (Fas,CD95) belong to the tumor necrosis (TNF) family, and play fundamental roles in regulating the immune system.The binding of FasL to Fas can lead to apoptosis of many cell types. Under normal circumstances, an increase in Fas expression on T cells is one of the control mechanisms that limit the immune response, thereby playing a vital role in the maintenance of immunological homeostasis and peripheral tolerance by clonal deletion of activated T lymphocytes. Many tumors have been shown to up-regulate FasL expression; however,when T cells are activated,they increase Fas expression on their cell surface. Accordingly,they become more sensitive to apoptosis when they interact with cells that express the FasL on their surface (Nagata et al., 1995; Ehrenschwender et al., 2009). Evidence for this mechanism of tumor immune privilege has been provided for several cancers (Zhou et al., 2006; Wu et al., 2010; Gomes et al., 2011; Cai et al., 2012; Tian et al., 2012; Wang et al., 2012) that have been shown to express FasL, including skin, lung, breast, and cervical cancers, gastric cancer. To date, the molecular events underlying the role of Fas/FasL in the progression to oral carcinoma are poorly understood (Chen et al., 1999).

In these cases, Fas and FasL may contribute to tumor immune privilege by inducing FasL-mediated apoptosis of host CTL and natural killer (NK) cells. TILs are difficult to be isolated and expanded in vitro, so we choose Jurkat T lymphocytes instead of TILs in our study. Jurkat is a Fas-sensitive cell line of human acute T cell leukemia and has been widely used experimentally as a model for activated

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T cells.Jurkat T cells express both Fas and FasL and have been used as a model system for apoptosis studies. More evidence indicates that some malignant cells express FasL, which can combine with Fas on activated T lymphocytes and induce T cells apoptosis. By this way, tumor cells counterattack host immune system and escape immune surveillance.

The goal of this study was to discover the relationship between Fas and FasL expression with the progression and apoptosis of OSCC. To this end, we compared the expression and apoptotic indices of the apoptosis-related antigens, Fas and FasL, in normal oral samples and oral squamous cancer.

Materials and Methods

Patients and controls

Informed consent was obtained from all individuals who participated in this study. The study group included 10 cases of normal oral epithelia and 38 cases of surgically excised OSCC. Of these, 18 patients had highly differentiated tumors (grade I according to WHO), 12 had moderately differentiated tumors (grade II), and one patient presented with a poorly differentiated tumor (grade III). 11 patients had lymph node metastasis. None of the patients had radiotherapy,chemotherapy, or any other interventional palliative or therapeutic measures prior to sampling. All specimens were fixed in 10% formalin and embedded in paraffin. Several representative blocks were selected and analyzed, most of which corresponded to the area of greatest tumor diameter. The serial sections were utilized for the present study.

Immunocytochemistry

Paraffin-embedded sections (4 μm) were immunohistochemically stained with rabbit-anti-FasL and rabbit-anti-Fas polyclonal antibodies, using an UltraSensitive TMS-P kit (ready-to-use, Maixin-Bio Co., FuZhou, China) and the streptavidin-perosidase (S-P) technique. Sections were deparaffinized in xylene, sequentially rehydrated in alcohol, and washed in phosphate-buffered saline (PBS). The sections were heated twice in a microwave for 5 min in citrate buffer (pH, 6.0) for antigen retrieval, and blocked in 3% H_2O_2 for 10 min to quench endogenous peroxidase. This was followed by incubation with nonimmune animal serum for 10 min, after which sections were incubated with primary antibody at 4 °C overnight, followed by washing with PBS. The sections were then incubated with secondary antibody for 10 min at room temperature, and washed again with PBS. Sections were developed with 3,3'-diaminobenzine (DAB) for 5 min, and counterstained with hematoxylin. Negative controls were incubated with PBS instead of primary antibody, and positive controls were provided by Maixin-Bio.

TUNEL analysis

DNA strand breaks were detected by TUNEL staining to identify apoptotic cells. The Apoptosis Detection Kit from Nanjing Jiancheng Bioengineering Institute was used. Staining was performed according to the manufacturer's instructions. In brief,tissue sections were deparaffinized, rehydrated through a series of graded alcohols, and washed in distilled water followed by PBS. Subsequently,the sections were permeabilized using proteinase K (20 μg/ml, 20 min at room temperature), washed in PBS.The slides were incubated with TUNEL reaction mixture for 60 min (humid chamber, 37 °C),and then washed twice in PBS. After multiple washes, the sections were treated with Converter-POD solution for 30 min (humid chamber, 37 °C), rinsed with PBS, and visualized by adding 3, 3'-diaminobenzine (DAB) for 10 min at room temperature. Sections were then washed in PBS, counterstained with hematoxylin, and finally,mounted for light microscopy. For negative controls,sections were incubated with label solution only (without terminal transferase) instead of the TUNEL reaction mixture.

Cell culture

The human OSCC cell line SCC-9 and the human T cell line Jurkat were obtained from ATCC(American type culture collection) and CCTCC(China Center for Type Culture Collection). SCC-9 was maintained with DMEM/F12 (1:1) (GIBCO, CA) and Jurkat cells with RPMI-1640 (Hyclone, CA), containing 10% FBS (Hyclone, CA), 100 I.U./ml ampicillin (Hyclone, CA), 100 μg/ml streptomycin (Hyclone, CA) in 5% CO_2 at 37°C. The medium was renewed every 3-4 days.

Apoptosis detection of T cell induced by FasL-expressing OSCC cells

2×10^6 OSCC cells were plated in 6-well plate, adhered for 24h. When the cell density reached 80% or above, OSCC cells were platedin 6-well plate, adhered for 24h. When the cell density reached 80% or above, the medium was thrown away and the cells were washed with fresh medium. Group A was experimental group: added Jurkat cellsat 4×10^5 cells/well to SCC-9 cells. Group B was the NOK-2 (BD Corporation) treated group: SCC-9 cells were incubated with neutralizing FasL monoclonal antibody NOK-2 at 10 μg/ml for 1h, and then the NOK-2 antibody was washed out, the same number of Jurkat cells were added to SCC-9 as in group A.Group C was negative control 1: Jurkat cells were cultured alone. Group D was negative control group 2: in which the same concentration of NOK-2 as Group B was added to Jurkat cells. Then, non-adherent Jurkat cells were collected carefully after co-cultured for 12 h and stained with propidium iodide (PI) and Annexin V- FITC conjugate (BestBio, Shanghai, China). Apoptosis of Jurkat cells was measured by FCM (Beckman Coulter, Inc.). A total of 10,000 events were acquired for each analysis. Each experiment repeated three times.

Semiquantitative analysis

Immunohistochemical staining: In each specimen, the staining intensity of Fas and Fas ligand of epithelial cells in normal oral samples, and OSCC were recorded by two independent observers in 10 adjacent high-power fields, and was scored on a scale from (-) to (+++), based on the proportion of positive tumor cells (nuclear or cytoplasmic staining for FasL and Fas).According to

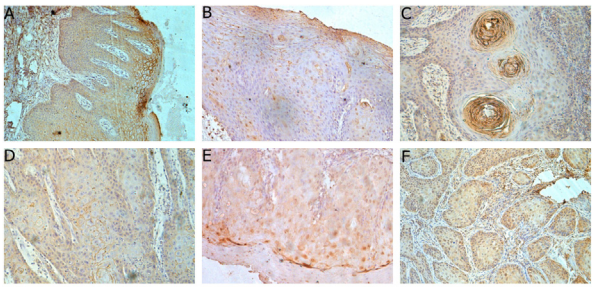

Figure 1. (A) Fas expression by immunohistochemistry of normal oral mucosa. (B) TILs stained with FasL antibody, 400×. (C) Immunohistochemical staining of TILs in oral squamous cell carcinoma with Fas antibody, 400×. (D)Photomicrograph part for expression of FasL in oral squamous cell carcinoma. 100×magnification shown. (E)Positive nuclear staining of TUNEL is seen in TILs of oral squamous cell carcinoma, 200×. (F) Positive nuclear staining of TUNEL is seen in oral squamous cell carcinoma. Original magnification, 200×

Hueber et al. (2002), a '(-)' score indicates the absence of any staining; a '+' score indicates positive staining in ≤ 25% of tumor cells; a '++' score indicates positive staining in > 25% but ≤ 50% of tumor cells; and a '+++' score indicates positive staining in > 50% of tumor cells. When there was a discrepancy in duplicate scores,the higher score was used. Uninterpretable scores (i.e., no tumor,absence of viable cells, or folded/lost tissue) were excluded from analysis. The Fas or FasL of TIL cells in OSCC were counted in the same way.

TUNEL staining: In each specimen,the number of total cells and apoptotic cells was recorded by two independent observers in 10 adjacent high-power fields. Positive apoptotic cells were cells with brown nuclei, The apoptotic index (AI) of each tissue specimen was calculated by counting the number of apoptotic cells and expressing it as a percentage.

Statistical analysis: Epidemiology Information (EPI) version 5.01 (US Center for Disease Control and Prevention, Atlanta, GA, and WHO) was used for data analysis. Fisher's exact test and χ^2 test were used to analyze the data. When the qualification of χ^2 test was not satisfied,continuity correlation was applied to modify the results.A t-test was used where appropriate after evaluation of distribution. All data are shown as mean ± SD. P values less than 0.05 were considered statistically significant.

Results

Frequency of Fas/FasL expression

In this study, Fas protein expression was observed in all normal oral mucosa. Immunopositive cells were mainly in the superficial layer of the epithelia, but were not in the stratum basale of normal oral mucosa (Figure 1A). Furthermore, staining of OSCC samples with Fas antibodies revealed low protein expression in several samples;the small amount of Fas expression that could be seen was localized to the center of the tumor. Most OSCC samples displayed no immunoreactivity and were scored as negative. Therefore, Fas-positive staining in normal oral mucosa samples had the highest scores,while staining in oral SCC samples scored significantly lower (Table 1). FasL expression was predominantly detected in the epithelial superficial layer of OSCC. FasL-positive staining was found in 23 of the 38 cases of OSCC; however, there was no FasL expression in any of the 10 normal oral mucosa cases. But there was also no correlation between FasL expression and tumor growth or histological type. In addition, FasL expression was significantly higher in OSCC than in normal mucosa (Table 1, Figure 1D).

Table 1. Frequency of FasL Expression in Normal Oral Mucosa and Oral Squamous Cell Carcinoma

Group	FasL				Positive No.(%)	*P* value
	—	+	++	+++		
NOM	10	0	0	0	0(0.0)	
OSCC	15	7	10	6	23(60.5)	<0.05*
grade I	8	3	5	2	10(55.5)	
grade II	4	3	3	2	8(66.6)	
grade III	3	1	2	2	5(62.5)	

NOM, normal oral mucosa; grade I, highly differentiated; grade II, moderately differentiated; grade III, poorly differentiated tumor; *compared to normal oral mucosa

Table 2. Relationship Between FasL Expression and the AI of Cancer Cells and TIL (mean+SD)

FasL	No.	AI	
		Tumor cells	TIL
Negative(-)	15	7.68±1.56	1.45±0.06
Positive	23	3.82±0.40	7.63±2.53
+	7	4.16±0.23	6.78±1.24
++	10	3.68±0.38	8.23±1.35
+++	6	2.93±0.32	10.5±2.14

Relationship between apoptosis and Fas/FasL expression

TUNEL methodology was used to quantify apoptosis. TUNEL analysis showed that positive apoptotic cells had brown nuclei. The apoptotic tumor-infiltrating lymphocytes (TIL) were smaller than the apoptotic tumor cells and were mainly confined to the tumor stroma. The results also indicated that the apoptosis-inducing function of OSCC and TIL is FasL-dependent, as apoptosis of FasL-negative cells was fairly higher than FasL-positive cells.In contrast, apoptosis of FasL-negative TIL was significantly less than that of FasL-positive TIL (P<0.05). The AI of tumor cells decreased and the AI of TIL increased in accordance with increasing FasL protein expression, indicating that there is a negative correlation between the AI of tumor cells and TIL (r=-0.72, P<0.05, Figure 1B, 1C, 1E, 1F, Table 2).

Relationship between Fas/FasL expression and lymph node metastasis

We analyzed the the relationship between FasL expression and lymph node metastasis, and found that FasL expression had a significant influence on lymph node metastasis compared to non-metastatic lymph nodes (P <0.05, Table 3). Furthermore, 11 of the metastatic lymph nodes were positive for FasL expression, but only two had Fas-positive expression.

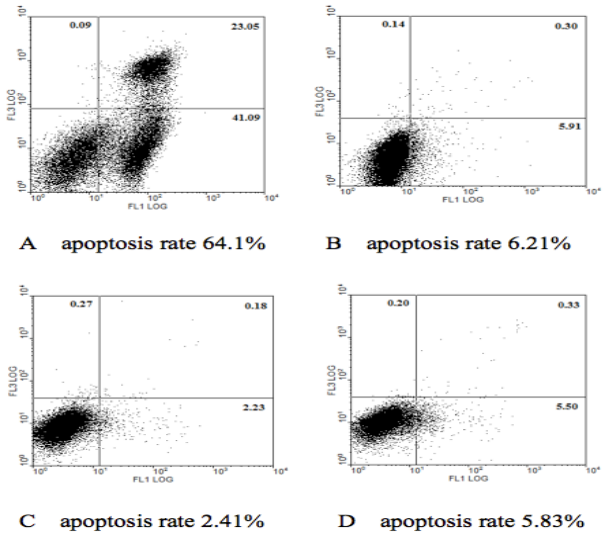

Figure 2. Apoptosis Rate (%) of Jurkat Cells Induced by OSCC Cells. Group A was experimental group. Group B was the NOK- 2 treated group. Group C was negative control 1: Jurkat cells were cultured alone. Group D was negative control group 2: in which the same concentration of NOK-2 as group B was added to Jurkat cells

Table 3. Relationship Between FasL Expression and Lymph Node Metastasis

Lymph node metastasis	No.	FasL expression -	+	Positive rate(%)	*P*
Metastasis	11	2	9	81.8	
Non-metastasis	27	13	14	51.9	<0.05

Apoptosis rate of T cells induced by FasL-expressing OSCC cells

In group A, after co-culture with SCC-9 cells, the total apoptotic rate of Jurkat cells was 61.5±2.44. It was significantly higher than that of group B (NOK-2 treated group), group C (negative control 1) or group D (negative control 2) was 6.32±0.56, 2.23 ±0.16, 6.11±0.42 respectively (Figure 2).

Discussion

It has been hypothesized that several types of malignant tumors change their expression of Fas/FasL in order to avoid harmful immunologic responses (Ibrahim et al., 2006). FasL is a membrane protein capable of inducing apoptosis in target cells upon coupling with its Fas receptor. The Fas-FasL system plays an important role in activated T cell apoptosis. Through induction of T cell apoptosis, the Fas-FasL system has been shown to play a role in eliminating harmful immunologic responses. Thus, the Fas-FasL pathway may, at least in part, play a role in tumor evasion of the immune system by inducing T cell apoptosis in tumor-infiltrating lymphocytes (TIL) (Hub et al., 1997; Kurita et al., 2010). In this report, both Fas(Data not shown) and FasL were found to be expressed in TIL and tumor cells. There was a close correlation between Fas and FasL expression in OSCC, and this constitutive expression suggests, as Zeytun et al. previously postulated (Zeytun et al., 1997), that tumor survival in the host depends upon which cell type can more efficiently induce apoptosis. The underlying theme in some studies (Younes et al., 2000; Ji et al., 2003) has been that the Fas-FasL system in malignant tumors is a normally occurring system that undergoes modification in the malignant counterpart. These modifications, which include increases in FasL expression, are advantageous towards the development of tumor growth and metastasis by allowing escape from immune surveillance and facilitating tissue destruction (Hueber et al., 2002). More to the point, the results of this study showed that FasL expression in OSCC tissues had statistically significantly higher expression of FasL compared to normal oral tissues. OSCC may escape immune surveillance when transformed cells fail to down-regulate FasL expression. According to the function of the Fas pathway, we presume that FasL expression in all OSCC groupsis indicative of the fact that the Fas-FasL system plays a role in facilitating tumor cell evasion from the immune system. Initially, this may allow a preinvasive lesion to develop into an invasive tumor by overwhelming the host's immune system (Chen et al., 1999; Zhou et al., 2006; Kurita et al., 2010). The extensive staining noted in the invasive tumors in this study also suggests that the Fas-FasL system may be one of the mechanisms that immunotherapy needs to overcome. We did find that the AI of tumor cells and of TIL cells varied with increasing FasL protein expression, in that, with increasing FasL expression, the AI of TIL cells increased, while the AI of tumor cells decreased, thereby demonstrating the negative correlation. Thus, these data suggest that an increase in FasL expression may correlate with the malignant progression of oral cancer. Fas and FasL-mediated apoptosis may serve as a proofreading mechanism in human neoplasia. As shown in Table 3, there was a significant positive correlation between FasL expression and lymph node metastasis in oral tumors, suggesting that FasL may be involved in the aggressive clinical behavior and invasive potential of the tumors, and thereby play an important role during oral tumor progression. Taken together, we propose that FasL may play a role in the processes of invasion and metastasis (Zielińska et al., 2008; Bozdogan et al., 2010). In conclusion, our study demonstrates that FasL expression is an early event in oral tumorigenesis, and thus may serve as a marker for early neoplasia and prognosis (Shimonishi et al., 2000). The observation of decreased FasL expression in invasive OSCC that are poorly differentiated, suggests that the extent of FasL expression may represent a balance between increased expression in response to neoplastic changes and decreased expression as squamous cells lose their differentiated phenotypes. An increased in FasL expression in OSCC compared to normal epithelium may represent very early OSCC changes in oral epithelium. So the results suggested that a change in Fas/FasL expression is an early event in oral tumorigenesis The identification of such early neoplastic changes may be useful in selecting patients for early interventional therapies, such as chemoprevention and/or nutritional therapies (Bennett et al., 1999; Koornstra et al., 2009; Hadzi-Mihailovic et al., 2009; Bozdogan et al., 2010; Gryko et al., 2011). Since information regarding the

Fas/FasL interaction in vivo in human cancers is extremely limited (Kassouf et al., 2008), the underlying mechanism of how tumor FasL contributes to treatment outcome in a specific type of human cancer needs further investigation. In summary, Fas/FasL signaling is involved in the growth of OSCC. This suggests that Fas/FasL-targeted therapy could potentially be used as a new approach to treat OSCC in its early stages. Of course, the numbers of tissue samples used is very low and hence future studies on a large cohort of patients are required.

Acknowledgements

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