SQUAMOUS CELL CARCINOMA OF THE LOWER LIP: FAS/FASL EXPRESSION, LYMPHOCYTE SUBTYPES AND OUTCOME

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Squamous cell carcinoma (SCC) of the lip is a relatively common malignancy of the head and neck region. Tumour thickness, grading and perineural invasion are significant prognostic indicators. However, there is still the need of new reliable biological markers able to predict the prognosis of the single cases with an unfavourable biological behaviour unpredictable by the classic clinical-pathological parameters. 32 cases of (SCC) of the lower lip were analysed for their clinicopathologic features, and immunohistochemical expression of Fas/FasL in neoplastic cells and in inflammatory infiltrate. Moreover the density and phenotype of tumour-infiltrating lymphocytes (TIL) were analysed. The results were related with the follow-up of the patients ranging from 2 to 6 years. The cases with over-expression of Fas/FasL in neoplastic cells and Fas+ in T cells preferentially showed a more aggressive clinical behaviour (P<0.01). Moreover we found an alteration of the normal expression of CD4 and CD8 lymphocyte types in ten cases. This data suggest that the Fas/FasL pathway is involved in the close relation between neoplastic cells and T cells and so in the biological behaviour of these tumours.

The most common type of oral cancer is squamous cell carcinoma, which accounts for approximately 90% of all oral malignancies. Therefore the oral cancer problem primarily concerns the diagnosis, biology and management of squamous cell carcinoma (SCC) (1). Squamous cell carcinoma of the lip is a common cancer of the head and neck area; its incidence is approximately one-quarter that for oral cancers. It occurs most frequently in the vermilion, particularly of the lower lip in elderly males. The main risk factors involved are prolonged life time exposure to sunlight and the use of tobacco and alcohol (2,3) .

The overall rate of recurrence was calculated as being 39.8%, and the determinate survival rate was found to be 72.9% after a 5-year follow-up in a large series of cases (4). Carcinoma of the lower lip can be treated primarily by surgical procedures. One of the most prominent characteristics of this tumour is that it can metastasize to sub-mental and sub-mandible lymph nodes. For that reason, bilateral suprahyoid dissection with surgical treatment of the primary lesion is a diagnostic approach for some investigators and therapeutic for others (5).

The risk of metastases to the sub-mandible and sub-mental lymph nodes in squamous cell carcinoma (SCC) of the lower lip is closely related to the primary tumour size and the differentiation

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of the tumour (6). However, the prognosis of same cases, with an unfavourable clinical behaviour, is unpredictable by the classic clinical-pathological parameters, probably because it is related to the bio-molecular characteristics of the single cases; therefore there is still the need of new reliable biological markers.

Apoptosis is a form of cell death that plays an important role during development, homeostasis, and in many diseases including cancer (7) . Host anti-tumour immunity is considered to play a role in protection against the development of malignancy and is related to the expression of Fas and its ligand (FasL) as recent reports have variously described (8,9) . So, we studied the expression of Fas/FasL in a selected series of 32 cases of SCC of the lower lip. Moreover, we also evaluated the relative density of lymphocytic infiltrates in the tumour, with particular attention to the CD4 and CD8 phenotype of TIL. The results were related with the follow-up and clinical-pathologic data of the patients. To the best of our knowledge, the importance of the Fas/FasL mechanism for the immune evasion by tumours provided a strong rationale for the examination of Fas/FasL expression and function in squamous cell carcinoma of the lower lip which is one of the most immunosuppressive human cancers (10).

MATERIALS AND METHODS

Selection of cases

Tumour specimens from 32 cases of SCC of the lower lip ($T_2N_0M_0$) were selected from the archive files of the Department of Biomorphological and Functional Sciences - Pathology Section, University of Naples Federico II. The cases were recruited consecutively from January 1994 to December 2000. The main prerequisite for inclusion in the study was the availability of a complete clinical follow-up of not less than two years. Informed consent was obtained from all patients. The study was performed in accordance with the guidelines of the institutional Ethics Committee. For each case, paraffin blocks from representative areas of the tumour were selected. Sections stained with haematoxylin-eosin were re-examined independently by two pathologists (SS, PS) to confirm the original diagnosis. Each case was subsequently evaluated jointly and discordance was resolved by consensus.

Immunohistochemistry

Immunohistochemical staining was performed on 4m-thick serial sections from formalin-fixed, paraffin-embedded tissue. A pre-treatment of sections with heat-induced antigen retrieval technique in a 650W microwave oven (3 sequential steps of 4 min each, in citrate buffer, pH 6.0, 10 mM) was performed. Then, incubation of sections for 20 minutes at room temperature with 0.3% hydrogen peroxide in methanol to quench endogenous peroxidases, and with non-immune horse serum (1:20, Dakopatts, Hamburg, Germany) diluted in PBS-bovine serum albumin (1%) for 25 minutes, to prevent non-specific immunostaining was carried out. After three washes with Tris-saline buffer, incubation was carried out overnight, in a moist chamber, at 4°C with the following primary antibodies: anti-Fas (C-20, Santa Cruz Biotechology, 1:200), anti FasL (N-20, Santa Cruz Biotechology, 1:200), anti-CD3 (UCHT-1, Dako, Hamburg, Germany, 1:50), anti- CD4 (DK 25, DAKO, Hamburg, Germany, 1:20) , and anti-CD8 (MT 310, DAKO, Hamburg, Germany, 1:50). Immuno-detection was performed with sequential 20 min incubations with biotinilated link-antibodies and peroxidase-labeled streptavidine (LSAB-HRP, Dako, Italy). As substrate, a chromogen solution of 3,3 diaminobenzidine (DAB, Vector Laboratories, Burlingham) with 0.3 % H_2O_2 was used. After nuclear counterstaining with haematoxylin, sections were coverslipped and mounted with synthetic medium (Entellan, Merck, Dermstad, Germany). As positive control sections of colon carcinoma had been used. Expression was scored semiquantitatively, as follows: 0 (<15% of cells with focal positivity); 1 (<30% of positive cells); 2 (>30% of positive cells); 3 (>60% positive cells).

Statistical analysis

The SPSS software (6.1 SPSS for Windows, Chicago, Illinois) was used for statistics. The inter-observer agreement on histological diagnoses was evaluated using k-statistics. Conventionally, a coefficient greater than 0.75 denotes excellent reproducibility, a value between 0.4 and 0.75 denotes moderate reproducibility and a value below 0.4 indicates marginal or poor reproducibility. Chi-square test or, when necessary, Fisher's exact test, was used for categorical variables. The Mann-Whitney U-test was used to evaluate differences among the average of the continuous variables. When comparing more than two groups of parameters, the Kruskal-Wallis-ANOVA one-way analysis of

variance was used. The non-parametric Spearman's coefficient was used to establish the significance among associations. Discriminant analysis was used to assess the ability of multiple markers to properly classify dead and alive subjects. Levels of statistical significance were set at $p< 0.05$.

RESULTS

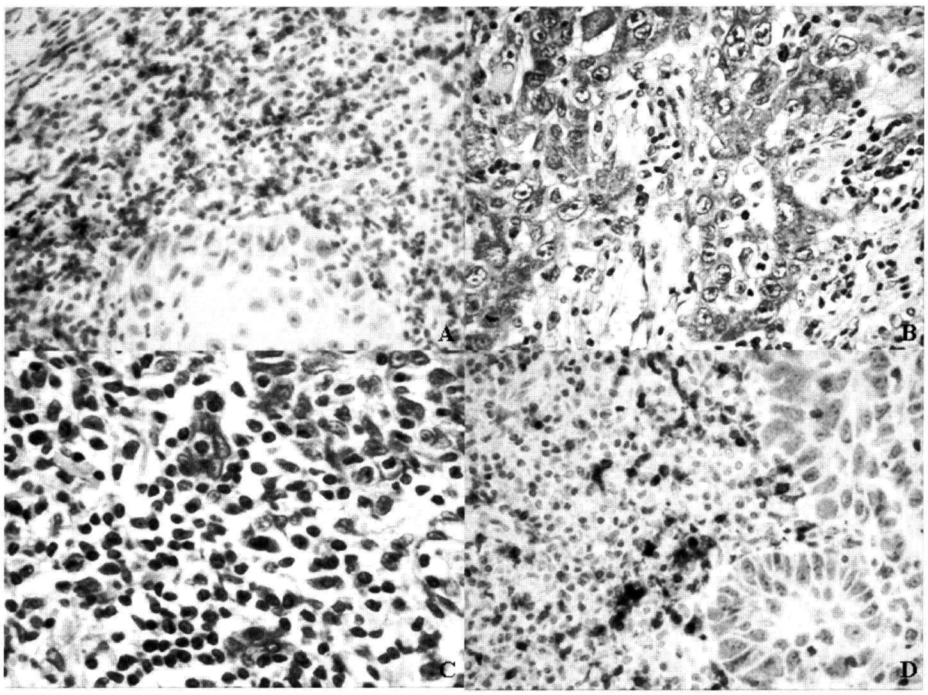
Study population

The mean age of patients was 64.2 years (range 41-88 years); 22 male and 10 female. A large excision was performed in 29 cases and a biopsy in 3 cases. All the tumours were clinically staged as $T_2N_0M_0$. The mean value of the largest tumour dimension (LTD) was 1.7cm (range: 1-3 cm). In all cases a diagnosis of squamous cell carcinoma was performed. Cytological atypia was graded as slight (+) in 9 cases; moderate (++) in 13 cases, and severe in ten cases. The mean value of mitotic index was 9 mitoses/10 HPF (range 3-20/ 10 HPF). The inter-observer reproducibility of the diagnosis was excellent (coefficient: 0.90). The mean follow-up time was 4 years (range 2-6 years). At the end of follow-up time 27 patients (84,37%) were alive, 23 without signs of disease, and four with lympho node metastasis. The remaining five patients (15,63%) died after respectively 24, 26, 29, 44 and 48 months after diagnosis for disease.

Immunohistochemistry

FasL expression (Fig. 1A-D) was detected in neoplastic cells of all SCC with overexpression (3) in seven cases. However, FasL-positive and

Fig. 1. *A): Intense expression of FasL in flogistic cells and negativity in tumour cells of a moderately differentiated lip squamous cell carcinoma characterized by favourable clinical behaviour and no lymph node metastasis (106x).*
B): Intense expression of Fas in tumour cells and moderate expression in flogistic cells in a lowly differentiated area of a lip squamous cell carcinoma characterized by favourable clinical behaviour and no lymph node metastasis (250x).
C): Moderate FasL expression in tumor cells of a lowly differentiated squamous cell carcinoma and no/low expression in flogistic cells of a squamous cell carcinoma characterized by a unfavourable clinical behaviour with lymph node metastasis (250x).
D): Intense expression of Fas in flogistic cells and moderate/low expression in cell tumours in case characterized by a unfavourable clinical behaviour with lymph node metastasis (106x).



FasL-negative areas were observed in all specimens. We determined that FasL expression was particularly strong in tumour areas closest to infiltrating lymphocytes and in tumours that contained substantial lymphocytic infiltrates. In addition, we observed that expression of Fas was heterogeneous in lower lip SCC. Moderate staining (score 2) for Fas was observed in some tumours, while other tumours contained few Fas+ neoplastic cells (score 1), and the Fas^+ cells were largely infiltrating lymphocytes, particularly in five cases. Moreover we found that the number of $CD4^+$ plus $CD8^+$ cells did not add up total CD3 +TIL, especially in the tumours with over-expression of FasL in the neoplastic cells.

There was no statistically significant correlation between Fas, FasL, CD4, CD8 expression, sex and age. There were significant correlation between Fas, FasL, CD4, CD8 expression, grading, lymph node metastases, clinical behaviour (Table I), and these differences were statistically significant ($p < 0.05$). Discriminant analysis output showed that by using a panel of the available markers (that were all statistically differently distributed in alive and dead patients) a 100% of sensitivity can be reached with a false positive rate of 7.4%.

DISCUSSION

Interactions between neoplastic cell and the host immune system have been a subject of interest. Today, the role of immune cells in the biological behaviour of human tumour remain undefined probably because a large range of conflicting data are available (11,13). Host anti-tumour immunity plays a role in protection against the development of malignancy. However, tumour evasion of the immune system, as low levels or lack of expression of tumour-associated antigens, various co-stimulatory or MHC molecules, and defective antigen processing, or production of immunosuppressive factors, determined the dysfunction of immune effector cells and accelerated the neoplastic development. Many evidence had accumulated indicating that immune cells in patients with squamous cell carcinoma are functionally impaired and this deficiency of T cells at the tumour site was attributed to effects of the tumour microenvironment (14-17). Moreover previously published results showed that Fas^+ lymphocytes constitute the major population of circulating lymphocytes in patients with SCC of the head and neck (9). More recently, identification of changes associated with proteolysis in TILs *in situ* focused attention on the possibility of apoptosis as explanation for TIL dysfunction. As reported, the presence of $TUNEL^+$ T lymphocytes in tumour tissues, in the absence of detectable apoptosis in tumour cells, indicated that many of the TILs were dying in the tumour microenvironment (18,19). Thus, tumour cells appear to be able to evade the host immune response by inducing death of immune cells present in the tumour microenvironment, particularly those immunocytes that are localized adjacent to or within tumour cell nests and in circulating T lymphocytes (20,21).

More recent evidence has suggested that Fas (Apo-1/CD95) ligand (FasL), one of the TNF family members, may be involved in the destruction of immune cells and in maintaining a state of tumour immune privilege (14,15,18,19,22). FasL is a type 2 membrane protein, which upon cross-linking its receptor, Fas, induces apoptosis of target cells, including activated lymphocytes (23). Whereas FasL expression on tissue cells, particularly at the immunologically privileged sites, is considered to be essential to eliminate the potentially tissue-destructive, activated T cells (24), its presence on all types of human neoplastic cells and its role in tumour-induced immune cell death has not been universally accepted (25). Although substantial concerns have been reported about the specificity of antibodies used for detection of FasL in human tissues (26-29), there is much reported evidence, that confirms in a series of *ex vivo* co-incubation experiments that tumour-associated FasL is indeed involved in inducing apoptotic signals in activated T lymphocytes (8-10). The presence of FasL on the tumour cell surface has always been confirmed by other group by RT-PCR for FasL mRNA, followed by Southern hybridization with FasL cDNA. The nominal "counterattack" of tumour against immune cells (18) depends by the expression on the neoplastic cells surface of functional FasL, which may in turn induce apoptosis of Fas-sensitive anti-tumour effector cells, thus rendering the tumour an immunologically privileged site. Tumour cell lines and tumours of various histological origin, including SCC, have been reported to express FasL (14,15,18,19,21,22,30,31).

Expression of both FasL and Fas in SCC *in situ* was heterogeneous: FasL-positive and -negative regions of the tumour were observed. This data are similar to that reported by Bennett *et al.* for esophageal carcinoma *in situ*. Furthermore, similar to Bennett *et al.* (21), we observed that the number of infiltrating TIL were significantly higher in the SCC of lower lip regions with strong FasL expression. These observations suggest that *in situ* FasL expression in tumour cells might be up-regulated by the presence of infiltrating lymphocytes. Because considerable numbers of tumour cells express FasL as well as Fas, it is likely that the tumour is relatively resistant to FasL expressed by itself or to FasL expressed on lymphocytes (32-35).

In our study, of the ten cases that presented the over-expression of FasL in the neoplastic cells and expression of Fas in TIL, four were characterized by lymph node metastasis respectively 25, 28, 29 and 32 months after the surgical treatment and in the remaining five cases the patients death respectively 24, 26, 29, 44, and 48 months after the original diagnosis for disease. Only in one case the over-expression of FasL in neoplastic cells was associated with a favourable biologic behaviour. Moreover, in these cases we found that the number of $CD4^+$ plus $CD8^+$ cells did not add up to total CD3 +TIL. Although the results of this study need to be confirmed on a larger series of cases, they suggest that the evaluation of Fas/FasL expression, particularly when combined with the above mentioned parameters may allow a post-surgical prognostic sub-typing of lower lip SCC, leading to the identification of tumours with different biological behaviour. Identification of the high-risk subclass of SCC of the lower lip characterized by the over-expression of FasL in the neoplastic cells, expression of Fas in TIL, and by the reduction of $CD4^+$ and $CD8^+$ lymphocytes in the tumour microenvironment, may be of primary importance in addressing appropriate therapeutic strategies, with closer follow-up protocols, to prevent and/or to delay relapses and metastases by using adjunctive, specifically targeted, more aggressive therapies.

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