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Original Paper

Metallothionein and Fas (CD95) are Expressed in Squamous Cell Carcinoma of the Tongue

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Metallothionein (MT) is a chelator present in myoepithelial cells, whilst the Fas-receptor (APO-1, CD95) has been described primarily in human T Jurkat cells. 20 cases of carcinoma of the tongue were investigated immunocytochemically with regard to MT, Fas and Bcl-2. In normal oral squamous epithelium, MT is located in the basal/parabasal dividing cells only. In well-differentiated nests of carcinomas, MT is observed almost entirely in peripherally located cells. *In situ* end-labelling indicates apoptosis in the centre of these nests, but not in the peripheral areas. Less-differentiated areas show more general MT-positivity, but little apoptosis. All 24 tumours are Fas-positive, but normal epithelia are mainly negative ($P < 0.0001$). Bcl-2 protein was sparse in the tumours compared with MT and Fas ($P < 0.0001$). We thus suggest that MT, possibly due to its chelating properties, may contribute to delaying cells entering apoptosis, both in normal epithelium near the base and in less-differentiated regions of carcinoma. Moreover, Fas may be present in cells of human malignancies, as well as those of established malignant cell lines. © **1997 Elsevier Science Ltd.**

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INTRODUCTION

APOPTOSIS, THE mechanism by which cells are naturally deleted within living tissues, has been shown to be controlled by the *Bcl-2* proto-oncogene. It encodes for a 26 kDa protein that protects cells from apoptosis [1, 2], thus prolonging their survival, which has been observed both in normal and neoplastic cells [3–7].

Two other proteins which could be involved in preventing apoptosis are metallothionein (MT) and Fas (APO-1, CD95), but few studies have investigated these proteins. MT has been found particularly in myoepithelial cells, but also in other cells [8–11], and has been found to be a marker of poor prognosis in breast cancer [12–14]. The Fas receptor has been demonstrated on certain fibroblasts and fibroblast-like synoviocytes, on certain skin components and on neuroblastoma tumour cells [15–18]. Ligation of the Fas ligand to the Fas receptor induces apoptosis in cultured human T Jurkat cells, B cells, rhabdomyosarcoma cells [19, 20] and in murine fibroblast L929 cells and T-cell lymphoma WR19L cells [21, 22].

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The aim of our study was to determine whether Bcl-2, MT and the Fas receptor are present in human squamous cell carcinoma of the tongue, one of the most common oral malignancies [23], and to examine whether, in normal squamous epithelium, MT is present only in the basal/parabasal layers, where cells are proliferative rather than apoptotic.

MATERIALS AND METHODS

Material

24 squamous cell carcinomas of the tongue were retrieved from archival material. Tumours from the base and from the tip of the tongue were excluded. All tumours were classified as T1N0M0 or T2N0M0, and the examined material constituted initial surgical specimens. The patients had not been exposed to any prior treatment for their tumour disease. There were 13 males and 11 females (mean age 60 years). The patients were followed for 3 years to observe local recurrence, regional and distant spread, as well as general outcome. In connection with six routine operations for obstructive sleep apnoea, normal non-neoplastic human uvulo-palatal specimens were obtained, and the squamous cell epithelia of their mucosae were used as controls.

Immunocytochemistry

From each case, 4–5 μm thick sections were cut from representative blocks of the original surgical specimen. One section was routinely stained with haematoxylin and eosin, and the others were placed on pretreated slides (poly-L-lysine) for immunocytochemistry. All slides were coded and the examiners had no access to clinical data.

Sections for immunocytochemistry (for all three antibodies; MT, Fas and Bcl-2) were treated with 3% hydrogen peroxide (H_2O_2) in distilled water to inhibit endogenous peroxidase activity, and then twice immersed in boiling citrate buffer, pH 6, in a microwave oven (26; two changes of 7 min each). After washing in tap and distilled water, the sections were subsequently incubated with 1:20 dilution of normal rabbit serum for 20 min at room temperature, followed by either 1:25 dilution of the monoclonal antibody to MT (Dako-MT, Clone E9); 1:25 for anti-Fas (Clone UB-2, Oncor, Gaithersburg, Maryland, U.S.A.); or 1:50 for Bcl-2 protein (Bcl-2, Dako, Copenhagen, Denmark) overnight at +4°C. Sections were then incubated with a 1:200 dilution of a biotinylated rabbit antiserum to mouse immunoglobulins for 30 min at room temperature, followed by a 1:100 dilution of the streptavidin–biotinylated peroxidase complex for 30 min, also at room temperature. Peroxidase activity was developed in diaminobenzidine (DAB) chromogene substrate.

Sections of cultured human T Jurkat cells were used as a positive control for Fas; breast tissue (containing myoepithelial cells) and normal and malignant parotid gland were used as a positive control for MT. For Bcl-2, a follicular lymphoma carrying the (t14;18) chromosomal translocation was used as a positive control. For all three antigens, tumour positivity was graded as 3+ if more than 75% of tumour cells were stained; 2+ if 25–75% of cells were stained; 1+ if <25% of cells were stained, unless all were negative. For Bcl-2, a tumour was only considered positive if the cytoplasmic or nuclear staining intensity was similar to that of the positive control. For all three antibodies, all available consecutive sections of the tumour were screened (between several hundred to thousands of cells). Negative controls, in which non-immune sera was substituted for the primary antibody, were also included.

In addition, as a means of assessing the proliferation of cells within the tissues, Ki-67 within the tissues was also examined (data not shown), immunostaining with the MIB-1 antibody (Immunotech France). Tumours were considered positive if more than 10% of cells were stained.

TUNEL analysis

After deparaffinisation, sections for the TUNEL (TdT dUPT Nick-End labelling) reaction were digested by 20 mg/ml Proteinase K for 15 min. After four washes in distilled water and quenching in 2.0% H_2O_2, the Apoptag® kit was applied according to the manufacturer's instructions (Oncor, Gaithersburg, Maryland, U.S.A.). TUNEL positive cells are considered to be apoptotic. Briefly, the TUNEL method is a tailing reaction where terminal deoxynucleotidyl transferase (TdT) catalyses a template-independent addition of deoxyribonucleotide triphosphate to the 3'-OH ends of single- or double (blunt ends)-stranded DNA. The incorporated nucleotides form a random heteropolymer of digoxigenin-11-dUTP and dATP, in a ratio that has been optimised for anti-digoxigenin antibody binding. The anti-digoxygenin antibody fragment carries a conjugated reporter enzyme (peroxidase) to the reaction site. Positive cells show a distinct nuclear staining. TdT was substituted with water for the negative control, and DNAase I was added (20 min at 37°C) after quenching in H_2O_2, thus producing DNA breaks in virtually all cells [27], to act as a positive control.

Statistics

The Wilcoxon test was used for comparison between the tumour expression of MT and Fas, between MT and Bcl-2, and between Fas and Bcl-2. The Mann–Whitney test was used for comparison of the expression of these parameters in tumours and that in control specimens. Finally, for the examination of any correlation between the expression of MT, Fas and Bcl-2, respectively, and clinical behaviour of tumours, Spearman's correlation test was used.

RESULTS

Of the normal epithelia examined, 5/5 were MT positive (one did not contain sufficient material for analysis), 3/6 were Fas positive and 1/5 was Bcl-2 positive. Unlike the other antigens, which were uniformly distributed throughout the epithelium, MT was localised only in the basal and parabasal cell layers (Figure 1(a)).

All tumours were morphologically moderately or well differentiated, although most also contained less differentiated areas. 22/24 were MT positive, all were Fas positive, and 5/24 were Bcl-2 positive. MT was generally localised in the peripheral cells of tumour nodules (Figures 1(b) and (c)), and there was some evidence of early invasion of the MT positive tumour cells from the basal areas of the epithelium into the mucosa (Figure 1(d)). TUNEL analysis of the tumour nodules showed that TUNEL-positivity was generally more pronounced in central rather than peripheral cells (Figure 1(e)). Fas positivity was uniformly distributed throughout tumour nodules, and did not show the peripheral localisation seen with MT (Figure 1(f)). Bcl-2 positive staining, in the few tumours which were positive, was uniform. MIB-1 positivity was also localised to the periphery of the tumour nodules, as seen with MT.

No difference between the expression of MT and Fas in tumours was observed ($P=0.58$), whilst MT as well as Fas were expressed significantly more than Bcl-2 ($P<0.0001$ and $P<0.0001$, respectively). MT and Bcl-2 were not expressed significantly more in tumours compared to normal epithelia ($P=0.41$ and $P=0.68$, respectively), whilst Fas was expressed significantly more in tumours than in normal epithelia ($P<0.0001$). Tests of the expression of the three different parameters (MT, Fas and Bcl-2) against the clinical outcome (Table 1) gave no significant correlation ($P=0.23$, 0.71 and 0.90, respectively).

Table 1. Immunoreactivity of normal and malignant epithelia of the tongue

	Tumour		Normal	
	positive	negative	positive	negative
MT	22	2	5*	0
Fas	24	0	3	3
Bcl-2	5	19	1	5

All cases which were considered positive were graded as either 2+ or 3+ for all antibodies. 3 patients died of their disease and another 3 had recurrence or spread to regional lymph nodes.

*For one normal specimen, there was insufficient material for analysis.

DISCUSSION

Apoptosis can be induced by a number of agents. Particularly in human T Jurkat cells, ligation of the Fas receptor is an important initial step in apoptosis [21,22]. Transduction signals can be hindered by certain proteins, for example, by the MT and Bcl-2 proteins. It is not known whether these proteins are involved *in vivo* in Fas-induced apoptotic death. Our demonstration of the chelator MT, the 'death' receptor Fas, but absence of the anti-apoptotic protein Bcl-2, in squamous carcinomas of the tongue are novel observations.

Metallothionein is present in a number of normal cells, particularly hepatocytes during fetal and early postnatal life, and myoepithelial cells [10–12,23]. The human genome contains two separate groups of genes, *MT-1* and *MT-2*, comprising 12 distinctive *MT* genes [29]. The gene products are cystein-rich proteins of low molecular weight (MW

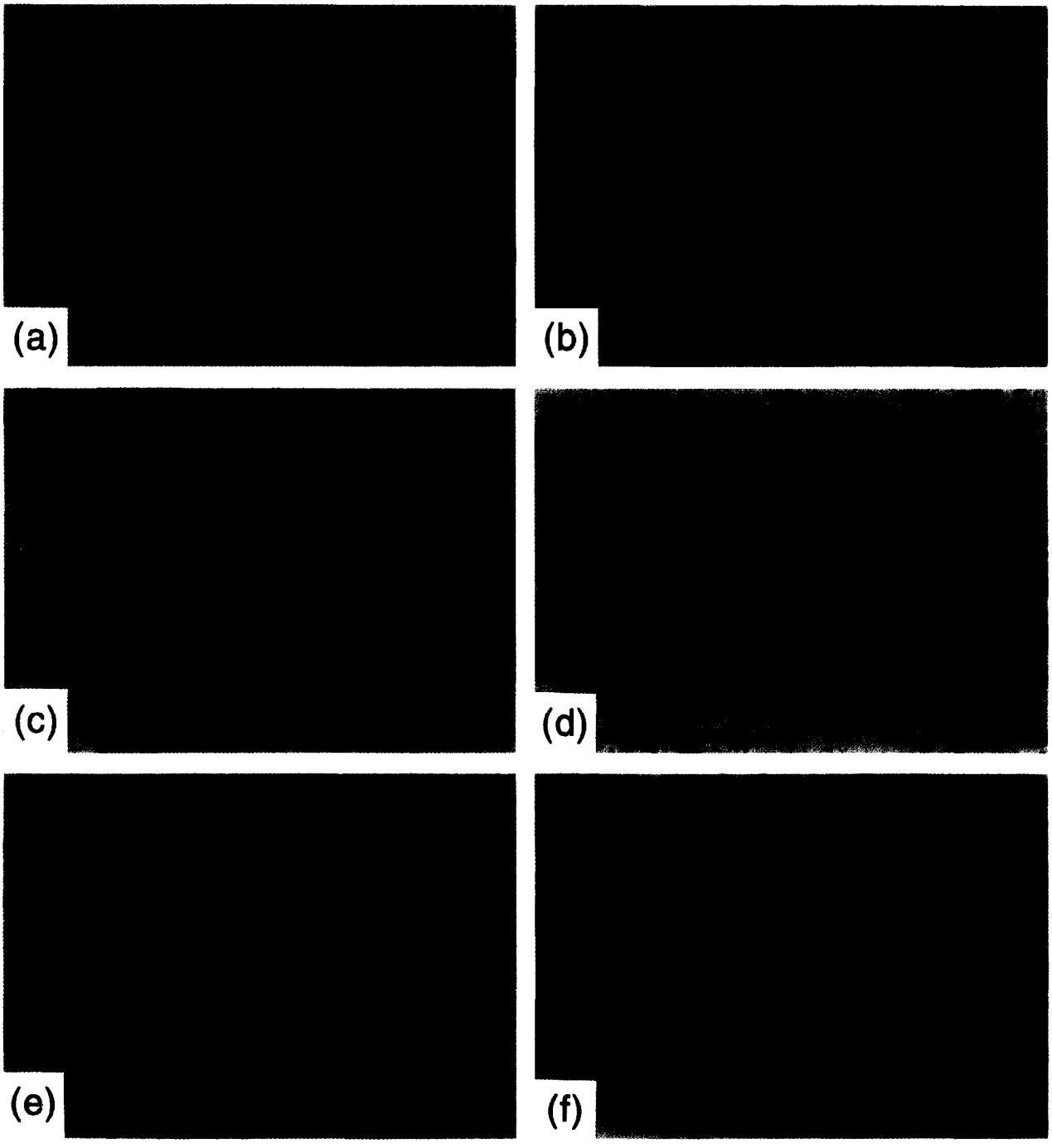

Figure 1. (a) Normal squamous cell epithelium from the oral mucosa. Metallothionein shows positive immunoreactivity in basal and parabasal cell layers only (× 170). (b, c) Well-differentiated squamous cell carcinoma with MT-positive cells at the periphery of tumour nests whilst cells in the centre of most tumour nodules were negative. One tumour nest is less differentiated but still MT-positive (lower) (b) × 130; (c) × 500. (d) Early invasion on an oral mucosa (top, E = epithelium), where the invasive proliferating cells and the basal/parabasal cells are MT-positive (× 130). (e) TUNEL-positive apoptotic cells are seen in the centre of the tumour nest, whilst there are no TUNEL-positive cells towards the periphery of the nest (× 340). (f) Tumour nests of squamous cell carcinoma showing an evenly distributed Fas-positivity in tumour cells (× 260).

6–7 kDa) with the capacity to bind several metals, for example, copper, zinc and cadmium. Metallothionein has a high sulphur content, and all 20 cysteine residues are involved in metal binding of seven bivalent heavy metal ions, giving rise to unique diamagnetic metal–thiolate clusters [4]. This ability to bind heavy metals (chelation) may interfere with intracellular redox balance. Theoretically, cystein-rich MT proteins could inhibit certain apoptotic transduction signals, for example, NFκ-B. Due to the chelating properties of MT, induction of MT synthesis *in vivo* in tumour cells is of interest. Our present investigation, which did not involve evaluation of the transcription of MT (reverse transcriptase–polymerase chain reaction, or *in situ* hybridisation of mRNA for MT) but merely detection of the protein revealed that MT was present in all tumours but two. In the normal squamous cell epithelium MT showed a very distinct pattern. The protein was in all cases located in the basal and in parabasal layers of the epithelia only (Figure 1(a)). These basal cells are viable and proliferating as opposed to apoptotic. In the more superficial areas of the epithelium, where the cells are more differentiated and closer to their ultimate death, MT was lacking. The basal/parabasal location of MT could thus imply that these cells are not apoptotic, possible due to protective chelating properties of MT. A particular peripheral staining pattern for MT was also observed in the tumours mimicking the basal staining pattern in normal epithelia (Figure 1(b, c)). If the arguments discussed above are valid, the presence of MT at the periphery could thus probably contribute to hindering these tumour cells from entering apoptosis. TUNEL positivity in the centre of the tumour nests but far less TUNEL-positive apoptotic cells in the MT-positive peripheral areas is in agreement with an ongoing apoptosis in the centre but less so in the periphery (Figure 1(d)). Likewise, the peripheral proliferation is also more pronounced than what could be registered in centrally located tumour cells (positive immunoreactivity for Ki-67, data not shown). Nevertheless, these novel data on MT are very interesting and call for *in vitro* experiments.

Fas was present in all 24 carcinomas, which differed significantly compared with normal squamous cell epithelium ($P < 0.0001$). To the best of our knowledge, Fas has never been demonstrated in human squamous carcinomas. Apparently, Fas is not expressed in normal oral squamous cell epithelium but in carcinomas. The presence of Fas receptors in tumours but not on the potential tumour precursor cells may indicate an upregulation of a receptor which already is present but inactive. Alternatively, a new transcription is induced. Should this kind of self-defence mechanism, i.e. activation of a 'death receptor', exist among tumour cells, exploration of whatever stimulus causing such an event would be of the utmost value.

The *Bcl-2* gene was originally discovered by Tsujimoto and associates, being involved in the majority of non-Hodgkin's B-cell lymphomas (t(14;18) chromosomal translocations) [19, 30, 31]. The *Bcl-2* gene (3.5 kb 5′ exon and 5 kb 3′ exon separated by a > 65 kb intron) encodes a 26 kDa protein. The Bcl-2-related protein family consists e.g. of Bcl-2 and Bcl-X_L (blockers), Bcl-X_S and bax (inducers), which tend to form dimers and/or heterodimers. The *bax* gene protein product ($\approx$ 21% homology to the Bcl-2 protein) can form heterodimers with Bcl-2 and thus abrogate its ability to suppress apoptosis [32]. The exact intracellular localisation of Bcl-2 is still debated, though it does appear to be membrane bound. Most of the protein is localised to cellular membranes in perinuclear cytoplasmic staining. Presumably this perinuclear membrane association corresponds to rough endoplasmatic reticulum and its continuum, the nuclear envelope [33, 34]. The exact anti-apoptotic mechanism of Bcl-2 remains unknown, though several suggestions have been put forward. It has been suggested that Bcl-2 protein functions to suppress lipid peroxidation and thus Bcl-2, at sites of free radical generation, might regulate an antioxidant pathway to prevent apoptosis [35]. High levels of Bcl-2 protein, aberrant patterns of Bcl-2 protein production, or both, have also been observed in a variety of solid tumours [24, 36–38]. A tumour of low-grade malignancy could thus be expected to express the Bcl-2 protein at a high level. In the present study only five tumours had a high level of Bcl-2 protein, whereas 19 tumours were devoid of immunoreactivity for the protein. Although no correlation with clinical outcome was found, the study revealed the apoptotic receptor Fas, the anti-apoptotic protein MT, and lack of anti-apoptotic Bcl-2 protein. The induction and function of these proteins are as yet not entirely known.

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