Loss of expression of TGF-ß1, TßRI, and TßRII correlates with differentiation in human oral squamous cell carcinomas

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Abstract. Despite advances in biological and molecular characteristics, the prognosis of oral squamous cell carcinomas is still very unfavourable and is based on the classical clinicopathological parameters. However, tumors with similar clinicopathological characteristics may differ dramatically in their clinical outcome. Thus, the identification of novel prognostic factors is necessary to improve prognostic and therapeutic approaches. Transforming growth factor-ß1 (TGF-ß1) is a potent growth inhibitor of epithelial cell proliferation, thus, inactivation of TGF-ß1 signalling may play a role in cancer. The expression levels of TGF-ß1 and its type I and type II receptors (TßRI and TßRII) were assessed by immunohistochemical and Western blot analyses in 22 oral squamous cell carcinoma lesions, in their normal adjacent mucosa and in the squamous carcinoma cell lines FaDu and CAL27. Immunohistochemistry on 22 oral carcinomas and case-matched normal oral mucosae demonstrated that TGF-ß1, TßRI, and TßRII were intensively and homogeneously expressed in all normal epithelia. In contrast, TGF-ß1 and its receptors were significantly reduced in poorly (G3) differentiated tumors as compared to moderately (G2) and well differentiated (G1) lesions ($p=2.8x10^{-3}$, $p=1.3x10^{-3}$, $p=2.8x10^{-3}$ and $p=1.3x10^{-3}$, respectively). The progressive reduction of the expression levels was confirmed by Western blotting. The oral squamous carcinoma cell lines Cal27 and FaDu demonstrated a reduced and a lack of TßRI expression, respectively. A significant decrease of TßRII expression, as compared to Cal27 cells, was shown in FaDu cells. Thus, the decreased expression of TßRII combined with the absence of TßRI could account for the resistance of FaDu cells to the growth-inhibiting effect of TGF-ß1. TGF-ß1 and TGF-ß1 receptor expression significantly decreased as tumors became less differentiated and thus more aggressive, suggesting a functional role of these molecules in oral tumor progression.

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Abbreviations: OSCC, oral squamous cell carcinoma; TGF-ß, transforming growth factor-ß1; TßRI, TGF-ß1 receptor type I; TßRII, TGF-ß1 receptor type II; ALK-5, activin receptor like kinase 5.

Key words: transforming growth factor-ß1, TGF-ß receptors, TßRI, ALK-5, TßRII, involucrin, oral squamous cell carcinoma, squamous carcinoma cell lines, tumor differentiation

Introduction

Oral cancer is the sixth most common malignancy and a major cause of cancer morbidity and mortality worldwide (1). Although there has been progress in the understanding of biological and molecular aspects and advances in cancer therapies, the overall survival has not improved (2).

Many molecular markers have been proposed for OSCC, however none shows a pivotal role for the assessment of individual prognosis in addition to the traditional clinicopathological evaluation.

Several studies demonstrated that proliferative activity of tumor cells, inactivation of tumor suppressor genes, and overexpression of growth factors may play a role in OSCC (3). Growth factors are involved in tumorigenesis in many different tissues, where they stimulate cellular proliferation, and modulate cell differentiation and apoptosis. However, growth factors do not only have the capability to stimulate cell growth, but they may also act as growth inhibitors, depending on the cell type. In fact, transforming growth factor-ß1 (TGF-ß1) may act as a growth suppressor in epithelial cells and as a growth stimulator in cells of mesenchymal origin.

TGF-ß1 is the prototype of a large family (ß1, ß2, ß3, ß1.2, and ß2.3) of cytokines that regulate a variety of cellular processes including cell proliferation, differentiation, motility, extracellular matrix production, angiogenesis and immune response (4). TGF-ß1, the most characterized isoform exhibits, along with TGF-ß3, stronger inhibitory effects than TGF-ß2.

TGF-ß1 generates cellular responses by interacting with specific TGF-ß receptors, named type I, type II, and type III. The type I and type II receptors (TßRI/ALK-5 and TßRII) are essential for eliciting the many effects of TGF-ß1, while the type III receptor (betaglycan) is involved in the presentation of the ligand to the type I and II signalling receptors (5). TGF-ß1 stimulation leads to intracellular signalling involving Smad activation (4). Evidence suggests that mutational inactivation of components in the TGF-ß1 signalling pathway contributes to the loss of TGF-ß1 growth control in cancer (6-8). However, the majority of human cancers preserve an apparently functional signalling system and become resistant to TGF-ß1 growth inhibition when no mutations are detected. In fact, increased TGF-ß1 levels are associated with poor prognosis in many types of cancers, enhancing tumor invasion via TGF-ß1 paracrine effects on tumor stroma, modulating angiogenesis and immunosuppression (9,10). On the other hand, overexpression of TGF-ß1 suppresses mammary tumorigenesis (11). Thus, these apparently contrasting effects highlight the dual role of TGF-ß1 in tumors: acting mainly as a growth inhibitor in early tumor stages, but contributing to tumor progression and invasiveness in more advanced diseases (12).

Previous studies on the role of TGF-ß1 and its receptors in OSCC tumorigenesis indicated that TßRII expression decreases as tumors become less differentiated and more aggressive, suggesting that aberrant TßRII expression may contribute to the pathogenesis of these tumors (13). Moreover, defects in post-receptor signalling may be involved in tumor development (14).

In order to understand the role of TGF-ß1 and its receptors TßRI and TßRII in oral cancer development, we analyzed TGF-ß1 and its receptors' expression patterns in 22 cases of OSCC, with different clinicopathological characteristics and compared to their histologically normal adjacent tissues. It has been shown that proliferation of cultured human keratinocytes is inhibited by lysophosphatidic acid (LPA) treatment that, by enhancing TGF-ß1 expression, induces terminal differentiation of the cells, as demonstrated by the increased expression of involucrin (15), a well-established marker of terminally differentiated keratinocytes. However, the relationship between TGF-ß1 and involucrin expression in keratinocyte differentiation is controversial since an inverse correlation in injured keratinocytes has been reported (16). The immunohistochemical expression of involucrin was analysed in parallel to monitor cellular differentiation.

Materials and methods

Patients and tissue samples. Primary oral squamous cell carcinomas and histologically normal oral samples were obtained from 22 patients prior to radiotherapeutic and/or chemotherapeutic treatments from the Department of Stomatology and Oral Science, University 'G. d'Annunzio' of Chieti-Pescara, and the Department of Pathology, University of Ancona, Italy. Informed consent was obtained from all patients. Tumors originated from gingiva (n= 6), tongue (n=7), palate (n=1), lip (n=2), cheek (n=3) and floor of mouth (n=3). The clinicopathologic characteristics of the tumors are summarized in Table I. The study was conducted according to the declaration of Helsinki and approval was granted by the Institutional Review Board.

Antibodies and cell lines. TGF-ß1 expression was evaluated using a rabbit polyclonal antibody (V, Santa Cruz, CA, USA) in the immunohistochemical analysis and an affinity purified polyclonal chicken antiserum (AF-101-NA, R&D Systems, Minneapolis, MN, USA) in Western blotting. Polyclonal rabbit antisera anti-TßRI (V-22, Santa Cruz), and anti-TßRII (L-21, Santa Cruz), were used in immunohistochemistry and Western blotting. The anti-human involucrin monoclonal antibody (SY5, Novocastra, Newcastle, UK) was used for immunohistochemistry. Normal rabbit and chicken sera were used as negative controls for the polyclonal antisera and a murine isotype identical monoclonal antibody for the anti-involucrin MAb. Anti-ß actin monoclonal antibody (clone AC-15, Sigma-Aldrich, St. Louis, MO, USA) was used to normalize loaded protein content.

The squamous cell carcinoma cell lines, Cal27, derived from tongue, and FaDu, derived from pharynx, were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum. CCL-64 mink lung epithelial cells were grown in DMEM containing 10% fetal calf serum. FRTL-5 rat thyroid cells were cultured as previously described (17).

Western blotting analysis. Protein extracts were prepared as previously described (17). The protein content was quantitated using BCA protein assay reagent (Pierce, Rockford, IL, USA). Protein extracts (20 μg/lane) were separated, under reducing conditions, by SDS-PAGE using a gradient 10-20% pre-cast gel. Proteins were then electrophoretically transferred to PVDF membranes (Bio-Rad Laboratories, Hercules, CA, USA) and blocked with phosphate buffered saline-Tween with 3% bovine serum albumin. Primary antibodies were incubated for 3 h at room temperature (R.T.) (dilutions: anti-TGF-ß1, 1:1500; anti-TßRI, 1:1000; anti-TßRII, 1:500; and anti-ß actin, 1:10000). Horseradish peroxidase-labelled rabbit anti-chicken IgY (1:1500 dilution; Promega, Madison, WI, USA), anti-rabbit IgG, and anti-mouse IgG (1:2000 dilution; Amersham Biosciences, UK) were used as secondary antibodies and the signal was visualized using the SuperSignal West Pico chemiluminescent substrate (Pierce). TGF-ß1 receptor expression ratios in tissues were determined by spot density measured with the Molecular Imager software (Bio-Rad Laboratories).

Immunohistochemistry. Immunohistochemical analysis was conducted by immunoperoxidase staining on serial sections (5 μm) of formalin-fixed paraffin-embedded tumors and normal epithelia using anti-TGF-ß1 (diluted at 1:200), anti-TßRI (diluted at 1:75), anti-TßRII (diluted at 1:20), anti-involucrin (diluted at 1:200), rabbit normal sera and isotype identical unrelated monoclonal antibody. Primary antibodies were incubated for 30 min at R.T. and specific immunoreactions were detected by a secondary biotinylated antibody and streptavidin-biotin-peroxidase complex (Super Sensitive immunodetection system kit, BioGenex, San Ramon, CA, USA). Peroxidase was developed by diaminobenzidine-hydrogen peroxide (DAB, BioGenex). Sections were counterstained with haematoxylin. All haematoxylin and eosin-stained

Table I. Clinicopathological characteristics of patients with oral squamous carcinoma.

Patient no.	Sex	Age (years)	Site	Grade of differentiation[a]	TNM[b]	Clinical stage[c]
1	F	55	Floor	G3	T1N2bM0	IVA
2	M	63	Tongue	G3	T2N1M0	III
3	M	67	Palate	G1	T2N0M0	II
4	M	62	Tongue	G2	T2N0M0	II
5	M	23	Tongue	G3	T2N1M0	III
6	M	42	Tongue	G3	T2N2bM1	IVC
7	M	48	Tongue	G2	T1N0M0	I
8	M	80	Lip	G1	T2N0M0	II
9	F	79	Gingiva	G2	T4aN0M0	IVA
10	F	75	Gingiva	G2	T2N2bM0	IVA
11	M	79	Gingiva	G2	T1N0M0	I
12	F	50	Cheek	G2	T1N0M0	I
13	M	59	Cheek	G3	T2N0M0	II
14	F	73	Gingiva	G1	T3N2bM0	IVA
15	M	67	Tongue	G3	T2N2cM0	IVA
16	M	63	Gingiva	G1	T3N0M0	III
17	M	74	Floor	G2	T2N1M0	III
18	M	51	Lip	G2	T1N0M0	I
19	M	77	Gingiva	G2	T2N0M0	II
20	M	91	Tongue	G1	T2N0M0	II
21	F	71	Floor	G1	T1N0M0	I
22	F	79	Cheek	G1	T1N1M0	III

[a]Prevalent degree of differentiation within a tumor lesion. [b]Tumor size: T1<20 mm; T2≥20-40 mm; T3≥40-60 mm; T4a>60 mm and invading adjacent structures. Lymph node involvement: N0, no regional lymph node metastases; N1, metastasis in a single ipsilateral lymph node (<30 mm); N2a, metastasis in a single ipsilateral lymph node (>30 mm and <60 mm); N2b, multiple ipsilateral lymph node involvement (<60 mm); N2c, bilateral or controlateral lymph node involvement (<60 mm). Distant metastases: M0, no distant metastases; M1, clinical evidence of distant metastases. [c]Clinical stage: I, T1N0M0; II, T2N0M0; III, T3N0M0 or T1/T2/T3N1M0; IVA, T1/T2/T3N2M0 or T4a, any N and M0; IVC, any T, any N and M1.

sections were reviewed and independently evaluated by two pathologists. Staining intensity was semiquantitatively classified as negative (-), weakly positive (+/- and +), and strongly positive (2^+ and 3^+).

DNA synthesis. To study the growth effects of TGF-ß1, cells were treated with different concentrations of TGF-ß1 (T1654, Sigma-Aldrich) for 24 h, pulse-labelled for 4 h with 1 μCi/ml of [^{3}H] thymidine (GE Healthcare Europe, Chalfont St. Giles, UK), fixed in 10% trichloroacetic acid for 15 min at R.T. and lysed in 2 N NaOH. Incorporation of [^{3}H] thymidine into DNA was measured by scintillation counting (Packard Instruments, Meriden, CT, USA).

Statistical analysis. The statistical significance of the association between TGF-ß1, TßRI, TßRII and involucrin levels of expression and the clinicopathological parameters of the tumors (age, sex, TNM status, grade of differentiation and clinical stage) was evaluated using Fisher's exact test. Values with $p \leq 5x10^{-2}$ were considered significant.

Results

TGF-ß1, TßRI, TßRII and involucrin immunohistochemical expression in oral squamous cell carcinomas. The expression of TGF-ß1, TßRI, TßRII and involucrin was evaluated on serial sections of neoplastic epithelium from 22 oral cancers and on patient-matched normal oral epithelium by immunohistochemical analysis. The immunohistochemical expression was evaluated according to criteria of both relative intensity and homogeneity of epithelial cell staining and correlated to the clinicopathological characteristics of the tumors summarized in Table I.

Fig. 1 illustrates representative TGF-ß1, TßRI, and TßRII immunostaining in well, moderately and poorly differentiated oral cancer tissues and in histologically normal oral epithelia adjacent to carcinomas. Normal mucosae exhibited a strong (3^+) and homogeneous expression of TGF-ß1, TßRI and TßRII, in contrast to poorly differentiated carcinomas (G3) showing only a weak (+/- and +) and heterogeneous immunostaining. In addition, it should be noted that intense and strong

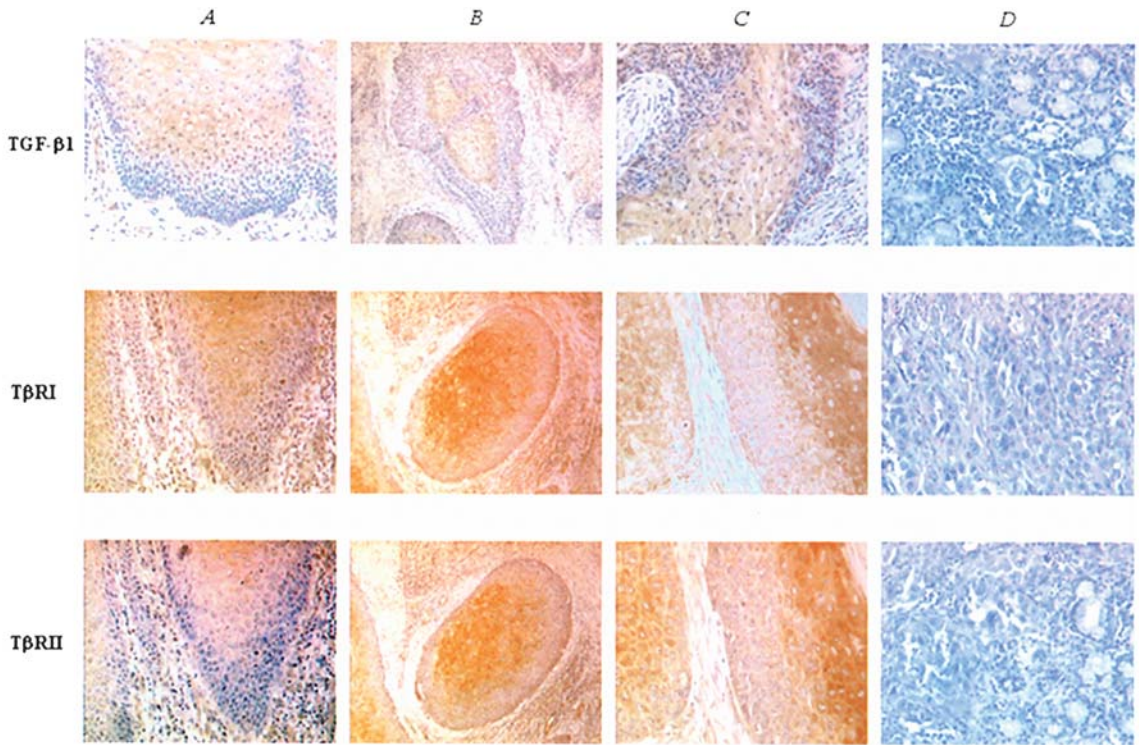

Figure 1. Immunohistochemical expression of TGF-ß1, TßRI and TßRII in normal oral epithelia and in oral carcinomas representative of different grading. A, normal epithelia; B, G1 tumors; C, G2 tumors; and D, G3 tumors. Immunoperoxidase counterstained with haematoxylin.

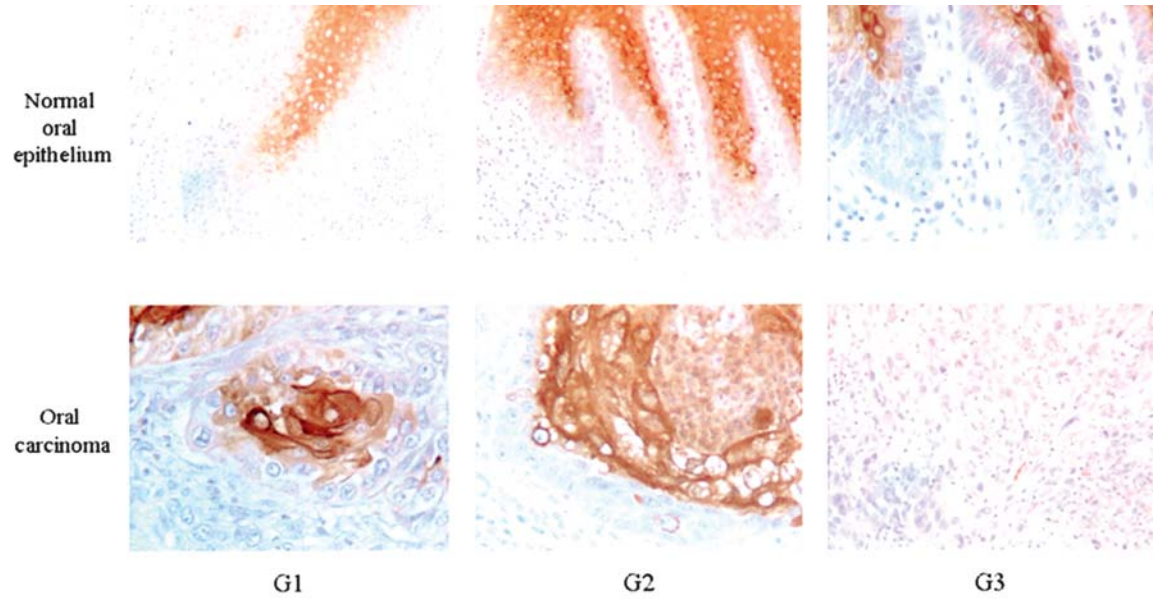

Figure 2. Immunohistochemical expression of involucrin in normal oral epithelium and in the corresponding oral carcinomas representative of different grading. Immunoperoxidase counterstained with haematoxylin.

(2^+ and 3^+) expression was paralleled by a diffuse positive immunostaining of >50% of epithelial cells. In contrast, weak expression (+/- and +) was associated to a focal heterogeneous and irregularly distributed expression in <50% of positive cells.

Overall, the TGF-ß1 and TGF-ß1 receptor immunostaining was similar in all normal mucosae tested, while in carcinomas the TGF-ß1 and TGF-ß1 receptor specific immunostaining, gradually decreased in intensity as carcinomas became less differentiated (Fig. 1 and Table II). In normal epithelium, the expression of TGF-ß1 was detected in superficial and intermediate epithelial layers, while it was negative in the basal layer. The tissue distribution and pattern of epithelial staining of TGF-ß1 and of the two receptors were essentially identical. By comparison, in tumors different immunostaining intensities were observed for the TGF-ß1 system molecules. In well

Table II. Immunohistochemical expression of TGF-ß1, TßRI, TßRII and involucrin in oral carcinomas and correlation with histological grading.

	G1 (n=7) Staining intensity			G2 (n=9) Staining intensity			G3 (n=6) Staining intensity			p-value[a]
	+/- / +	2^+	3^+	+/- / +	2^+	3^+	+/- / +	2^+	3^+	
TGF-ß1	0	2	5	4	5	0	6	0	0	$2.8x10^{-3}$
TßRI	0	0	7	1	8	0	5	1	0	$1.3x10^{-3}$
TßRII	0	2	5	4	5	0	6	0	0	$2.8x10^{-3}$
Involucrin	0	0	7	1	8	0	5	1	0	$1.3x10^{-3}$

Staining intensity: +/- and +, weak; 2^+, intense; 3^+, strong. [a]TGF-ß1, TßRI, TßRII and involucrin weak vs intense and strong expression in G1 and G2 carcinomas vs G3 carcinomas.

differentiated tumors (G1), the staining intensity was scored as strong (3^+) and was similar to that of normal epithelium. The expression levels of TGF-ß1, TßRI and TßRII progressively decreased in G2 and G3 carcinomas. The loss of immunostaining was significantly associated to high-grade carcinomas (G3) as compared to moderately (G2) and well differentiated (G1) lesions ($p=2.8x10^{-3}$, $p=1.3x10^{-3}$, $p=2.8x10^{-3}$ and $p=1.3x10^{-3}$, respectively). TGF-ß system molecule expression did not correlate significantly with other clinical parameters including age, sex, tumor size, TNM status, lymph node status and clinical stage.

It is noteworthy that the immunostaining intensity did not vary within the tumor lesion, with the exception of the neoplastic invasive front, observed in most sections, that was in all cases negative suggesting that more malignant cells had lost TGF-ß system growth control. The co-localization of TGF-ß1, TßRI and TßRII molecules, analyzed in serial sections, supports the cooperative role of these molecules in the pathogenesis of OSCC (Fig. 1) suggesting an autocrine source for TßRI and TßRII activation.

In parallel, involucrin immunohistochemical expression was analysed in all sections (Fig. 2 and Table II). Immunohistostaining intensity for involucrin was inversely correlated with tumor grade. Weak involucrin expression was significantly associated with high-grade tumors as compared to well and moderately differentiated carcinomas ($p=1.3x10^{-3}$). Indeed, G3 tumors displayed in 5/6 cases only weak (+/- or +) immunostaining, while G1 tumors in all cases (7/7) demonstrated very intense (3^+) protein expression. The anti-involucrin staining was consistently reduced (- or +/-) at the invasive front, thus confirming the progressive loss of involucrin expression in the more aggressive cell population.

TGF-ß1, TßRI and TßRII protein expression in tissue extracts from OSCC patients. Human oral tissues were analyzed by Western blotting using an anti-TGF-ß1 antibody recognizing mature TGF-ß1 as well as the precursor form. Under reducing conditions, TGF-ß1 precursor protein (50 kDa) was detected in all normal tissues analyzed (18). In contrast, gradually decreasing amounts of TGF-ß1 precursor were detected in tissue extracts derived from tumors characterized by progressively higher differentiation grade and stage. Immunoblots were

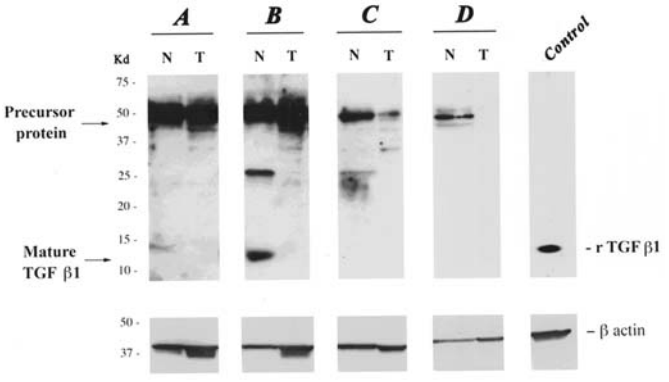

Figure 3. TGF-ß1 protein expression in representative oral normal and neoplastic tissues from patients with different grading and clinical stage by Western blot analysis. Molecular mass standards in kDa on the left. N, adjacent normal oral tissue. T, tumor tissue. Control, 5 ng recombinant porcine TGF-ß1 were loaded as control. Bottom panel, ß actin expression to normalize protein loading. A, grade 1, stage I; B, grade 1, stage II; C, grade 2, stage III; and D, grade 3, stage IV.

probed with protein ß actin antibody to normalize the amounts of loaded proteins. Pair-wise analysis revealed that in oral tissues at early stages of malignancy, and well-differentiated tumors (G1), TGF-ß1 precursor protein levels were similar in neoplastic and normal tissues (Fig. 3A and B). These levels gradually decreased in advanced stages (III and IV stage tumors) compared to the corresponding normal tissues, in association with decreasing levels of cellular differentiation (G2 and G3, respectively) (Fig. 3C and D).

The mature reduced TGF-ß1 molecule (12 kDa) was detected in the normal counterparts of G1 (stage I and II tumors), while it was absent in G2 and G3 tumors (stages III and IV) (Fig. 3). Additional bands representing unreduced mature TGF-ß1 (25 kDa) were detected in normal tissues of some patients (Fig. 3, patients B and C), but not in the corresponding neoplastic tissues. It is interesting to note that while TGF-ß1 was detected both as reduced and unreduced forms in the normal tissue from patient B (G1, stage II), it was evident only in the unreduced 25-kDa TGF-ß1 form in the normal tissue of patient C (G2, III stage). Therefore, the significant differences of TGF-ß1 expression in G2 and G3

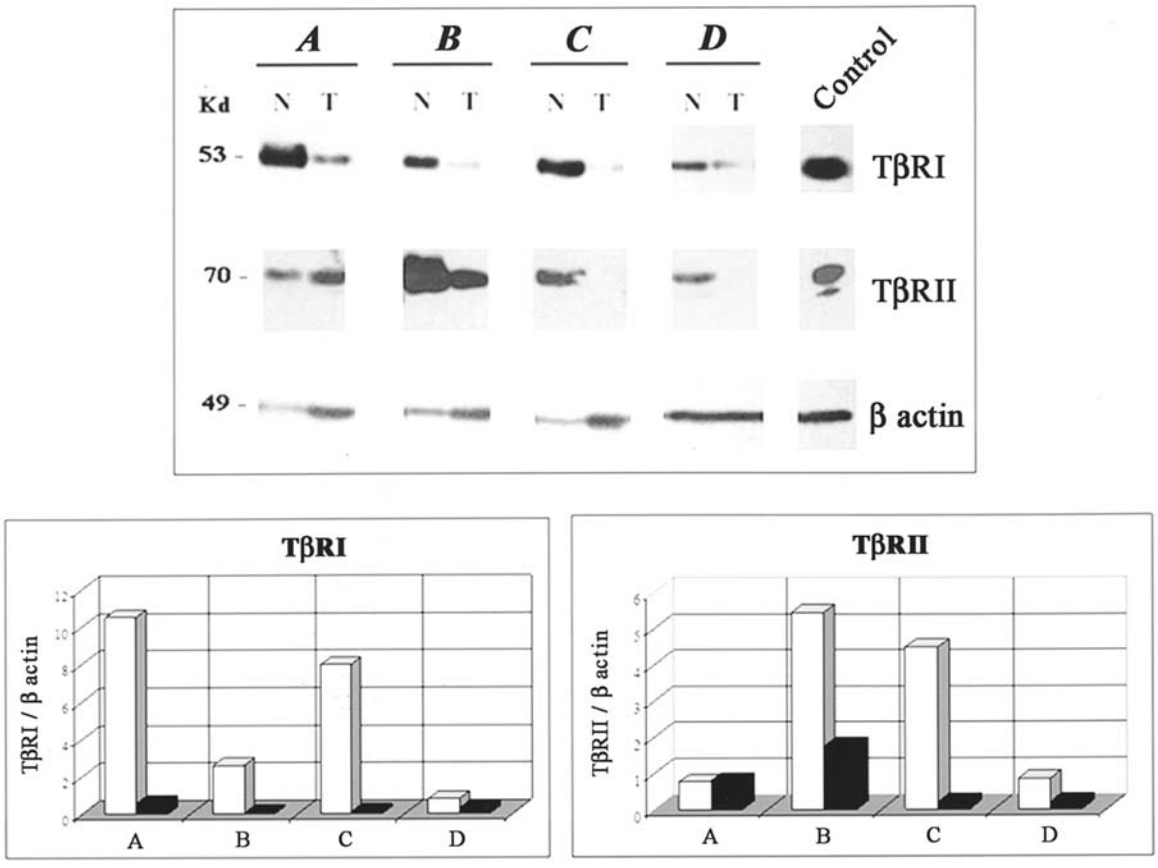

Figure 4. Representative examples of TßRI and TßRII protein expression assessed by Western blot analysis in adjacent oral normal (N) and cancer tissue (T). Anti-ß actin antibody was used to verify equal loading. Molecular mass standards are given in kDa on the left. Control, FRTL-5 cell lysate used as positive control for TßRI and TßRII expression. Scale bar: white bars, normal; black bars, tumor. Panels illustrate the ratio of the integrated densities of TßRI and TßRII, respectively, to the housekeeping gene ß actin in the pairs of normal and neoplastic tissues. A, grade 1, stage I; B, grade 1, stage II; C, grade 2, stage III; and D, grade 3, stage IV.

advanced stage tumors as compared to their corresponding adjacent normal tissues and to G1 early-stage carcinomas further strengthen the inverse correlation between TGF-ß1 levels and progression towards more advanced and aggressive neoplasms. In fact, while G1 stage I and II tumors tended to have similar or slightly diminished TGF-ß1 protein levels compared to their corresponding normal tissues, G2-stage III and G3-stage IV tumors demonstrated a significant and progressive reduction of TGF-ß1 expression levels associated to the progressive loss of cell differentiation (Fig. 3).

Western blot analysis revealed reduced TßRI and TßRII protein levels in tumors as compared to the corresponding normal tissues. The ratio of the integrated density between the tested molecules and the housekeeping protein ß actin, was used to compare the protein amount in the pairs of normal and malignant tissues. Fig. 4 illustrates representative TßRI and TßRII expression analyses in the same patients as shown in Fig. 3. The normalized densitometric analyses indicated dramatic reductions of TßRI in the tumor samples compared to the normal controls in all cases and stages (Fig. 4). The tumor tissues of patient A (G1-stage I) and patient C (G2-stage III) showed the most significant reduction in TßRI expression, compared to their normal counterparts. In contrast, the ratios of normal:tumor were only slightly decreased (by 3- to 4-fold) in patients B and D, which showed significantly lower TßRI levels in their normal tissues (Fig. 4).

Comparable levels of TßRII expression were observed in normal and tumor tissues from patient A (G1-stage I) while patient B (G1-stage II) showed a reduction of TßRII expression in tumor tissue compared to adjacent normal tissue. The tumor tissues of the G2-stage III patient showed a strong reduction in TßRII expression compared to corresponding normal tissues. Paralleling TßRI, also TßRII protein levels were reduced in G3-stage IV tumor tissue by 3-4 fold as compared to normal counterparts (Fig. 4).

Effect of TGF-ß1 on DNA synthesis of squamous carcinoma cell lines in relation to their basal TßRI and TßRII expression. Squamous carcinoma cell lines, Cal27 and FaDu derived from tongue and pharynx, respectively, were treated with TGF-ß1 (concentrations from 5 to 15 ng/ml) to measure DNA synthesis by [^{3}H] thymidine incorporation. TGF-ß1 (at 5 ng/ml) strongly interfered with DNA synthesis (90% inhibition) in the control mink lung epithelial cell line CCL-64 (19) and in FRTL-5 rat thyroid epithelial cells (20) (55% inhibition) (data not shown). The treatment with 5 ng/ml of TGF-ß1 weakly inhibited cell growth of the human cell line Cal27, while higher concentrations (10 and 15 ng/ml) stimulated cell proliferation in a dose-dependent manner (Fig. 5). Interestingly, TGF-ß1 had no significant effect on cell proliferation of the human squamous carcinoma cell line FaDu. Thus, distinct TGF-ß1 effects on the proliferation of the two squamous carcinoma cell lines

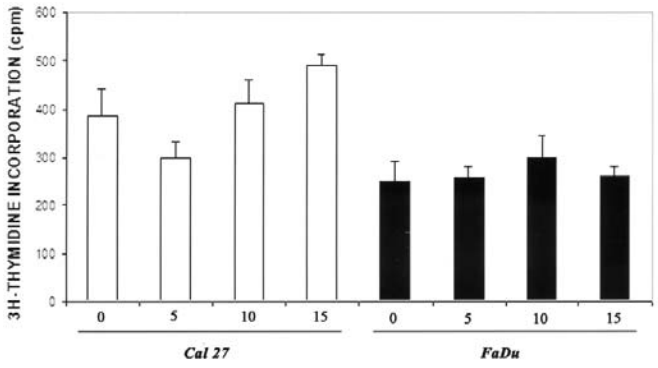

Figure 5. Proliferation effects of TGF-ß1 on squamous cell carcinoma cell lines. DNA synthesis was measured by [^{3}H] thymidine incorporation after 24-h treatment with TGF-ß1 at 5, 10 and 15 ng/ml. 0, untreated proliferating cells. Experiments were performed in triplicate and at least three times.

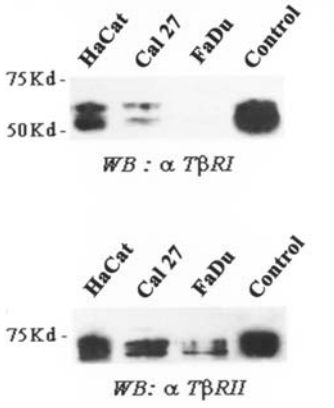

Figure 6. TßRI and TßRII protein expression assessed by Western blot analysis in Cal27 and FaDu squamous carcinoma cell lines and in the human immortalized keratinocyte cell line HaCaT. Control, the rat thyroid epithelial cell line FRTL-5 was used as positive control.

analyzed were observed. Since a decreased receptor expression is considered one of the mechanisms responsible for the loss of TGF-ß sensitivity, the expression patterns of TßRI and TßRII molecules in Cal27 and FaDu cell lines were analyzed by Western blot analysis. The FRTL-5 rat thyroid epithelial cell line, expressing both TßRI and TßRII (21), served as control. Our results showed a reduced expression of TßRI in Cal27 cells and a complete lack of expression in FaDu cells as compared to the HaCaT cell line used as a human immortalized keratinocyte control (Fig. 6). In addition, a significant decrease of TßRII expression, as compared to Cal27 and HaCaT cells, was shown in FaDu cells (Fig. 6). Thus, the decreased expression of TßRII and the absence of TßRI, could account for the resistance of FaDu cells to the growth-inhibiting effect of TGF-ß1.

Discussion

TGF-ß1, TßRI, TßRII and involucrin expression was evaluated in neoplastic tissue and histologically normal adjacent epithelium from 22 oral cancer patients by immunohistochemical and Western blot analyses. The expression levels were compared and correlated with the clinicopathological characteristics of the tumor to determine whether particular expression patterns relate to specific tumor characteristics. The findings herein demonstrated that TGF-ß system molecules and involucrin were intensively and homogeneously expressed in all normal epithelia surrounding the tumors. In contrast, a decreased expression of TGF-ß1, TGF-ß1 receptors and involucrin was observed in the majority of neoplastic tissues. The reduction of the TGF-ß1, TGF-ß receptors and involucrin levels correlated with the tumor grading, decreasing progressively from G1 to G3 tumors ($p=2.8x10^{-3}$, $p=1.3x10^{-3}$, $p=2.8x10^{-3}$ and $p=1.3x10^{-3}$, respectively). No correlation was found with other clinicopathological parameters.

TGF-ß1 is a potent inducer of differentiation for normal epithelial cells and its expression is increased in terminal differentiation of mucosal keratinocytes (22). According to several authors, human keratinocytes treated with TGF-ß1 do not proliferate, but differentiate as monitored by an increased level of involucrin, induced by the extracellular matrix protein ßig-h3 and transglutaminase expression through the PI3K/Akt signalling pathway (15,23). During malignant transformation, keratinocytes acquire several characteristics including resistance to the TGF-ß1 growth inhibitory effects combined to a reduced ability to undergo terminal differentiation.

The loss of responsiveness by tumor cells can be obtained by different mechanisms, involving alteration in growth factors, receptors and/or signal transduction molecule levels and/or activation. Evidence supporting the role of TßRII as a tumor suppressor protein includes inactivating mutations (24) as well as targeted deletion of the TßRII molecule (25). Mutations or deletions of TßRI have also been reported, but they are uncommon (26).

In oral cancer structural defects of TßRI (26) and TßRII as well as transcriptional down-regulation (27) abrogating TGF-ß1 signalling have been described. The decrease of TßRII by down-regulation has been established in metastatic lesions as compared to the primary tumors, and in primary tumors as compared to the normal epithelium, thus strongly implicating TßRII in the disease progression (28). Moreover, previous studies on the role of TGF-ß1 and its receptors in OSCC tumorigenesis have indicated that TßRII expression decreases as tumors become more aggressive (13).

TGF-ß1 is secreted as an inactive precursor complex LAP-TGF-ß1, consisting of a dimer of TGF-ß1 associated with a dimer of ß1-latency-associated peptide (ß1-LAP) (29). TGF-ß1 activation implies the releasing from the ß1-LAP dimer. Although latency appears to be a critical step in the control of TGF-ß1 activity, the quantitative relationship between latent and active TGF-ß1 levels is not clear, since enhanced latent TGF-ß1 expression does not always correlate with increased levels of active TGF-ß1 (30,31).

The progressive loss of TGF-ß1 immunohistochemical expression observed in higher-grade tumors was confirmed by Western blotting, since immunohistochemistry did not discriminate active or latent TGF-ß1 forms. Our results showed that the expression of the precursor LAP-TGF-ß1 gradually decreased from G1 oral squamous cell carcinoma to high-grade tumors (G3). Moreover, in all cases tumor tissues expressed lower levels of LAP-TGF-ß1 than the matched adjacent normal tissues. The general reduction of the precursor TGF-ß1 levels in the tumor tissues may reflect a low level of mature TGF-ß1

protein. In fact, active TGF-ß1 was detected by Western blotting in normal epithelium derived from patients with G1 tumors displaying high levels of precursor protein. It is noteworthy that no mature TGF-ß1 protein was detected in tumor specimens by Western blotting. On the other hand, the lack of immunodetection of mature TGF-ß1 in normal adjacent epithelia from advanced stage tumors suggests the possibility of a permissive role of normal tissues allowing tumor invasion and progression. In fact, low levels of TGF-ß1 in tumor cells can account for the relative resistance to the TGF-ß1 growth inhibitory activity, while low levels in the surrounding normal epithelium could facilitate tumor invasion. In addition, the significant reduction of TßRI and TßRII expression in human OSCC of high grade as compared to low-grade carcinomas and to normal epithelia demonstrate the progressive reduction of the growth controlling TGF-ß1 system.

The effect of TGF-ß1 was evaluated on squamous carcinoma cell lines by [^{3}H] thymidine incorporation. The growth of Cal27 cells treated with TGF-ß1 was inhibited at 5 ng/ml and stimulated, in a dose-dependent manner, by higher concentrations. In contrast, TGF-ß1 was ineffective in the growth inhibition of FaDu cells, as previously reported (32). It is interesting to note that, although Cal27 cells expressed reduced levels of TßRI and TßRII receptors, when compared with non-neoplastic human keratinocytes, they were still able to respond to TGF-ß1 in terms of growth inhibition at low doses or stimulation at higher doses. This result underlines the complexity of TGF-ß1 signalling and its apparently contrasting cellular effects that could be mediated by cross-talk with other growth factors and by activation of different signal transduction pathways (33,34).

In contrast, the TGF-ß1-resistant cell line FaDu displayed a complete loss of TßRI receptor and a decreased expression of TßRII as compared with normal human keratinocytes and Cal27 cells. These results indicate that a critical minimal ratio between type I and II receptor expression is required for TGF-ß1-mediated growth inhibition. However, Qiu *et al* (35) demonstrated in FaDu cells the complete loss of expression of SMAD4 protein due to homozygous deletion of the gene (36). Therefore, the altered TGF-ß1 receptor pattern in FaDu cells contributes to, but does not totally account for TGF-ß1 resistance.

In conclusion our study demonstrates that the decrease of TGF-ß1, TGF-ß receptor and involucrin expression, observed in all cases, represents a common event in OSCC and is significantly related to the progressive loss of cellular differentiation. These observations suggest the possibility that down-regulation of the TGF-ß1 system plays a key role in the progression of oral squamous cell carcinomas and its detection could be an additional and useful diagnostic or prognostic tool.

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