Inhibition of the Transforming Growth Factor-β/Smad Signaling Pathway in the Epithelium of Oral Lichen

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The basal cells in epithelium of the erythematous form of oral lichen display hyperproliferation compared with normal oral mucosa. In this study we examined whether this is associated with disrupted production, activation, or signal transduction of the epithelial growth inhibitor transforming growth factor (TGF) β1. In situ immunostaining showed that most epithelial cells in normal oral mucosa had nuclear and cytoplasmic Smad4 and phosphorylated Smad2/3, but expressed little or no Smad7. Expression of latency-associated peptide TGF-β1, latent TGF-β binding protein 1, TGF-β type I receptor, and TGF-β type II receptor was readily seen, but only very little TGF-β1 was activated. In erythematous oral lichen, basal and lower spinous epithelial layers showed staining for latency-associated peptide TGF-β1, TGF-β type I receptor, and TGF-β type II receptor. A band with scanty staining for these molecules, but with marked staining for active TGF-β1, was seen in the upper spinous and granular layers. Numbers of epithelial cell nuclei with Smad4 and phosphorylated Smad2/3 staining were significantly reduced in erythematous oral lichen compared with normal oral mucosa. Basal and suprabasal cell layers in erythematous oral lichen showed strong cytoplasmic Smad7 protein staining, but in spinous and granular layers Smad7 was localized to the cell membrane. In situ hybridization showed strong Smad7 mRNA expression in almost all basal keratinocytes in erythematous oral lichen; by contrast, no or occasionally very weak Smad7 mRNA expression was seen in these cells in normal oral mucosa. The observations indicate that inhibition of the TGF-β/Smad pathway may account for the hyperproliferation of keratinocytes in erythematous oral lichen. Key words: epithelial proliferation/keratinocytes/oral mucosa. J Invest Dermatol 121:00–00, 2003

O ral lichen (OL), which includes oral lichenoid reactions and oral lichen planus (OLP), is a common oral disease affecting about 2% of the populations examined (Axell and Rundquist, 1987; Salonen et al, 1990). Oral lichenoid reactions are defined as adverse reactions to dental materials, whereas changes with unknown etiology are termed OLP (Boleswska et al, 1990). Histologically, oral lichenoid reactions and OLP are indistinguishable from each other (Boleswska and Reibel, 1989). The lesions are characterized by a typical band-like mononuclear inflammatory infiltrate in the connective tissue, dominated by T lymphocytes (Hedberg et al, 1986). Pathologic changes in the epithelium include hyperthorakeratosis or parakeratosis, acanthosis, atrophy, and liquefaction degeneration of basal cells (Andreasen, 1968; Kramer et al, 1978). Clinically, OL shows white papular, reticular, erythematous, plaques, and ulcerative forms (Andreasen, 1968; Kramer et al, 1978). Erythematous OL (ERY OL) is one of the common forms of the disease and is often associated with oral discomfort in the affected persons (Andreasen, 1968).

In order to maintain the stratified epithelium's normal anatomy and thereby its functions, there is a fine-tuned balance between the continuous proliferation of basal keratinocytes and maturation and cell death of terminally differentiating keratinocytes (Gandarillas, 2000). Epithelial proliferation and differentiation are regulated by an intricate signaling network of different peptides (cytokines/growth factors) produced by keratinocytes themselves, stromal cells, and infiltrating inflammatory cells and their respective receptors (Dotto, 1999; Freedberg et al, 2001). Transforming growth factor β (TGF-β) has been shown to have important regulatory functions in epithelial proliferation and differentiation (Roberts, 1998; Ten Dijke et al, 2002). Among the three TGF-β isofoms, TGF-β1 is known as the most prominent regulator. TGF-β1 inhibits epithelial cell growth through transcriptional repression of the growth promoting gene c-myc (Pietenpol et al, 1990), and upregulates the cyclin-dependent kinase inhibitors p15 and p21 (Hannon and Beach, 1994; Datto et al, 1995). This leaves the retinoblastoma protein in a hypo-phosphorylated state and thereby retains the cells in the G1 phase (Weinberg, 1995). TGF-β1 can also induce apoptosis in keratinocytes (Min et al, 1999), and affect keratinocyte differentiation by upregulating the expression of integrins and keratins (Mansbridge and Hanawalt, 1988; Jiang et al, 1995; Zambrouno et al, 1995). The crucial role of TGF-β1 for epithelial development is highlighted in studies on transgenic mice: the epidermis of TGF-β1-null mice displays marked hyperproliferation (Glick et al, 1993), whereas mice with targeted overexpression of active TGF-β1 exhibit severely disrupted skin development, leading to neonatal death (Sellhayer et al, 1993).

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Abbreviations: ERY OL, erythematous oral lichen; LAP-TGF-β1, latency-associated peptide TGF-β1; LTBP-1, latent TGF-β binding protein 1; NOM, normal oral mucosa; OLP, oral lichen; OLP, oral lichen planus; TBR1, TGF-β type I receptor; TBR2, TGF-β type II receptor.
TGF-β1 is predominantly secreted as a large latent protein complex, consisting of the mature (active) TGF-β1 homodimer, the latency-associated peptide (LAP), and the high molecular weight latent TGF-β binding protein (LTBP) (Wakefield et al., 1988; Miyazono et al., 1991). Extracellularly, latent TGF-β1 is proteolytically cleaved with the release of active TGF-β1. On the target cell, active TGF-β1 binds to a hetero-tetrameric complex of two transmembrane signaling receptors: TGF-β type I (TβRI) and TGF-β type II (TβRII) receptors (Eben et al., 1993). TβRII phosphorylates TβRI upon binding of TGF-β1 (Wrana et al., 1994) and TβRII subsequently phosphorylates either of two intracellular protein homologs, Smad2 or Smad3 (Nakao et al., 1997b). Phosphorylated Smad2 and Smad3 associate in the cytoplasm with Smad4 and the resulting complexes translocate to the nucleus, where they modulate transcription in collaboration with other coactivators and corepressors (Massague, 2000). This signaling pathway, however, can be blocked by binding of Smad7 to the intracellular domain of the TβRII, which inhibits the phosphorylation of Smad2 and Smad3 (Nakao et al., 1997a).

We have previously reported that ERY OL lesions show epithelial atrophy and hyperproliferation of basal keratinocytes compared with reticular OL and normal oral mucosa (NOM) epithelium (Karatsidis et al., 2003). From studies on transgenic mice with null expression of Smad3, or active TGF-β1 constitutively targeted to the keratin 10 promoter, or overexpression of Smad7 (Cui et al., 1995; Ashcroft et al., 1999; He et al., 2002), it is known that interference with the TGF-β/Smad pathway can cause such changes. In this study, we show that this pathway is inhibited in ERY OL because, concomitantly with an increased activation of TGF-β1, there is an increased Smad7 expression and membrane translocation, and significantly reduced nuclear accumulation of Smad2, Smad3, and Smad4 in the epithelium of ERY OL. Parallel to the findings in transgenic mice, this thus may be the cause for the epithelial hyperproliferation in ERY OL.

MATERIALS AND METHODS

Specimens

Biopsies were taken, after informed consent, from volunteers with ERY OL (n = 12) or with NOM (n = 11). No distinction was made between OLP and oral lichenoid reactions. Clinical diagnosis was made by an experienced clinician (TA), and pathologic diagnosis was confirmed on hematoxylin and eosin sections of the biopsies. The clinical and histopathologic criteria used were those described by Kramer et al. (1978). Buccal mucosal biopsies were taken from sites that typified the clinical diagnosis and character of the lesion. The volunteers were tested for oral Candida infection using Dentocult dip slides (Orion Diagnostica, Espoo, Finland) and the biopsies were examined by PAS staining. None of the patients included in this study showed Candida infection at the time of biopsy. Biopsies were snap-frozen on dry ice (−70°C), oriented, and embedded in OCT compound (Sakura Finetek, Tokyo, Japan), and stored at −80°C. Biopsies for in situ hybridization were fixed in 4% buffered formaldehyde. Five micron thick sections were cut at 20°C in a cryostat and slides were stored at −20°C until used. The study was carried out according to the Helsinki Declaration’s principles for biomedical research and approved by the Ethical Committee of Health, Oslo, Norway.

Immunohistochemistry

Antibodies against the following proteins were used in this study: the mature and active TGF-β1 (mouse IgG, 1.25 μg per ml, BioSource International, Camarillo, CA), LAP (TGF-β1) (goat IgG, 1.3 μg per ml, R&D, Oxfordshire, UK), LTBP-1 (rabbit IgG, 0.3 μg per ml, a kind gift from Dr C.-H. Heldin, Ludwig Institute for Cancer Research, Uppsala, Sweden), keratinocyte transglutaminase (mouse IgG, 0.45 μg per ml, Biomedical Technologies, Stoughton, MA). The following antibodies from Santa Cruz Biotechnology (Santa Cruz, CA) were used: anti-TGF-βRI (rabbit IgG, 0.3 μg per ml), anti-TGF-βRII (rabbit IgG, 0.3 μg per ml), phosphorylated Smad2/3 (rabbit IgG, 0.25 μg per ml), Smad4 (mouse IgG, 2 μg per ml), Smad7 (goat IgG, 4 μg per ml), plasminogen activator inhibitor 1 (PAI-1) (mouse IgG, 1 μg per ml).

Single stainings were performed after fixation of cryosections in 4% paraformaldehyde for 20 min, followed by 2 × 5 min washes in phosphate-buffered saline (PBS) and preincubation with 0.3% H2O2 for 30 min. Sections were rewash and incubated in 5% of appropriate normal serum for 30 min (the species of the normal serum used was equivalent to the species of the secondary antibody used; see below). Primary antibodies were diluted in PBS with 1% bovine serum albumin, applied on the sections, and incubated overnight at 4°C. Subsequently, the sections were washed in PBS and incubated 30 min with the appropriate secondary biotinylated antibody preparation (horse antirabbit IgG, goat antirabbit IgG, or rabbit antigoat IgG, all diluted 1:200 and all from Vector Laboratories, Burlingame, CA). After washing in PBS, sections were incubated with avidin—biotin complex conjugated with horseradish peroxidase (Vector Laboratories) for 30 min, washed, and finally developed with 3,3′-diaminobenzidine as substrate (Sigma-Aldrich, St Louis, MO).

In double stainings, the sections were fixed as above, washed in PBS, and incubated in 10% normal goat serum and 10% normal horse serum for 30 min. Primary antibodies for keratinocyte transglutaminase and LTBP-1 were diluted and incubated for 1 h. Sections were washed again in PBS followed by incubation of a secondary goat antirabbit biotinylated antibody preparation (1:200, Vector Laboratories) for 30 min. After washing, the sections were incubated in Cy3-labeled streptavidin (1:800, Amersham Pharmacia Biotech, Uppsala, Sweden) for 30 min. Sections were again washed, followed by sequential incubation with avidin (10 μg per ml) and biotin (1 μg per ml), both from Sigma-Aldrich, for 10 min each, and an incubation of a secondary horse antirabbit biotinylated antibody preparation (1:200, Vector Laboratories) for 30 min. This was followed by incubation with Cy2-labeled streptavidin (1:800, Amersham Pharmacia) for 30 min. Sections were finally washed and counterstained with DAPI (Molecular Probes, Eugene, OR). Isotype-matched control antibodies showed no staining with the immunohistological detection techniques used.

In situ hybridization

After fixation in 4% buffered formaldehyde solution for 24 h, biopsies were processed through graded alcohols, oriented, and embedded in paraffin. Four micron thick paraffin sections were cut and mounted on polystyrene-coated glass slides. Prior to staining, sections were dewaxed and dehydrated according to standard procedures. Endogenous peroxidase activity was quenched with 3% H2O2 in methanol. The sections were then rehydrated with distilled water and equilibrated in TE-buffer (10 mM Tris–HCl, 1 mM ethylenediamine tetraacetic acid, pH 7.6). Permeabilization was performed with protease K (Sigma-Aldrich, 20 μg per ml in TE-buffer, 30 min, 37°C). Proteolytic activity was stopped by incubation with glycine in PBS (2 mg per ml). Sections were postfixed in 4% paraformaldehyde in PBS for 15 min. Acetylation was done with freshly prepared triethanolamine buffer (100 mM, pH 8) with 0.5% acetic anhydride (2 × 5 min). Equilibration and prehybridization were carried out with 5 × sodium citrate/chloride buffer (SSC) (10 min, room temperature) and hybridization buffer (Dako, 2 h, 65°C). The biotinylated (152 bp) or radiolabeled radiolabeled (152 bp) transcripts, sections were incubated for 18 h at 52°C with biotinylated antisense or sense probe (GreenStar Biotin oligonucleotide probe; antisense, 48 bp hybridizing to nucleotides 440–487 in the coding sequence of human Smad7; GeneDetect, Auckland, New Zealand) diluted in hybridization buffer (1 μg per ml). Stringency washes were 2 × SSC (1 h, 52°C), 2 × SSC diluted with 50% formamide (20 min, 52°C), 0.1 × SSC (20 min, 52°C). Blocking was done with Tris-buffered saline (TBS; 0.1 M Tris–HCl, pH 7.5, 0.15 M NaCl) with 1% casein (TBS-C). Further incubations and washings were then peroxidase-conjugated rabbit antibody antibodies (Dako P506, 1:100 in TBS-C, 30 min), washing with TBS with 0.05% Tween 20 (TBS-T), biotinyl–tyramide (Dako GenPoint kit K628; 60 min), washing with PBS-T, alkaline phosphatase-conjugated rabbit antibody antibodies (Dako D5807; 175 in TBS-C, 30 min). The sections were finally developed with BCIP/NBT substrate (20 min) and counterstained with Nuclear Fast Red. All aqueous solutions for in situ hybridizations were prepared with DEPC-distilled water.

Cell countings

Images of sections were captured with a digital camera using objectives with 20 ×, 40 ×, 60 ×, or 100 × magnification and monitored on a 17-inch computer screen by means of an imaging software program (Soft Imaging System, Münster, Germany). Cell countings were performed using objectives with 20 × magnification. For ERY OL specimens, the field chosen for counting was located over the inflammatory cell infiltrate. Total numbers of keratinocytes and numbers of immunohistochemically stained nuclei were counted in the entire part of the epithelium visible on the computer screen.

Statistics

t tests were used to compare differences between groups. Differences were considered to be statistically significant at p < 0.001.
RESULTS
An overview of the stainings is presented in Table I.

In NOM, basal, spinous, and granular cell layers showed perinuclear, nuclear, and cytoplasmic staining for LAP-TGF-β1 that gradually decreased in intensity from the spinous to the outer epithelial surface (Fig 1a). Cytoplasmic staining for LTBP-1 was seen in all epithelial cell layers in NOM, and this was strongest in spinous, granular, and superficial cell areas (Fig 1c). Speckled staining for active TGF-β1 was observed in upper spinous and granular layers (Fig 1e with enlargement, showing arrow-marked spots). Strong confluent staining for active TGF-β1 was seen in the outermost superficial epithelial cell layer in NOM (Fig 1c). TβRI and TβRII expression was present as a nuclear, a cell membrane, and a cytoplasmic staining in all NOM epithelial cell layers (Fig 1g, i). In NOM epithelium, the staining was most intense in basal cell layers for both TβRI and TβRII. The majority of the cells throughout the epithelium showed nuclear and cytoplasmic staining for both phosphorylated Smad2/3 and Smad4 (Fig 1b, m, respectively). NOM epithelium showed areas with patched weak or no cytoplasmic staining for Smad7 (Fig 1a with enlargement). In situ hybridization showed only very weak and scattered nuclear and perinuclear Smad7 mRNA in basal keratinocytes of NOM (Fig 1q, antisense). Control hybridization (sense) did not show any staining (Fig 1r).

In ERY OL, basal and lower spinous layers showed cytoplasmic staining for LAP-TGF-β1, which was especially intense around cell nuclei (Fig 1b with enlargement). Typically, a band-like area with only scattered or absent LAP-TGF-β1 staining was observed in the uppermost spinous and granular cell layers (Fig 1b with enlargement). There was strong cytoplasmic staining for LTBP-1 in basal and lower spinous cell layers (Fig 1d). In upper spinous and granular epithelial layers, this staining had a marked pericellular and intercellular location (Fig 1d with enlargement). Active TGF-β1 consistently showed a clear cell membrane/pericellular staining in the granular and upper spinous epithelial layers, but not in the basal cell layers in ERY OL epithelium (Fig 1f with enlargement). This band of active TGF-β1 staining was complementary to the cell layers that displayed decreased LAP-TGF-β1 staining (compare Fig 1b, f with respective enlargements). The reduced stores of LAP-TGF-β1 may therefore be a direct result of depletion of latent TGF-β1 in these layers as a consequence of increased activation. Cell membrane, cytoplasmic, and intense perinuclear staining for TβRI and TβRII was seen (Fig 1h, j, respectively). This staining was strongly reduced or absent in granular and in upper parts of the spinous epithelial cell layers, often in a band-like pattern, similar to that described above for LAP-TGF-β1. Phosphorylated Smad2/3 (Fig 1l with enlargement) and Smad4 (Fig 1n with enlargement) stainings revealed significantly reduced numbers of cells with nuclear and/or cytoplasmic staining among keratinocytes in all epithelial layers in OL compared with epithelial cells in NOM (Table II, p<0.001, t test). The Smad7 staining pattern in ERY OL was profoundly altered compared with NOM (Fig 1p with enlargement). In contrast to the nearly absent Smad7 expression in NOM, basal and suprabasal cell layers in ERY OL showed strong cytoplasmic Smad7 staining. In spinous and granular layers, the Smad7 staining was particularly localized to the cell membrane. Almost all basal keratinocytes showed strong nuclear and perinuclear expression of Smad7 mRNA in ERY OL epithelium (Fig 1i, antisense). Control hybridization (sense) did not reveal any staining (Fig 1l).

LTBP-1 has been demonstrated to be targeted to elements of the extracellular matrix through cross-linking by the enzyme transglutaminase (Nunes et al., 1997; Verderio et al., 1999). Double staining for LTBP-1 and keratinocyte transglutaminase in NOM epithelium showed that LTBP-1 was localized preferentially in the cytoplasm and keratinocyte transglutaminase in cell membrane/pericellular epithelial areas (Fig 1u). In contrast, double staining for LTBP-1 and keratinocyte transglutaminase in ERY epithelium showed a cell membrane/pericellular codistribution of these molecules in spinous and granular cell layers (Fig 1v−v3).

Finally, we carried out staining for PAI-1, which is upregulated by TGF-β through Smad (Dennler et al., 1998). NOM epithelium showed ample cytoplasmic and pericellular staining in spinous and granular cell layers (Fig 1ur). In contrast, there was very little staining for PAI-1 in ERY OL epithelium, and this was restricted to only scattered epithelial cells (Fig 1x).

In sum, TGF-β1 was strongly activated in parts of the ERY OL epithelium, but the signal transduction through Smad appeared to be blocked by Smad7. This was accompanied by a concomitant decreased expression of PAI-1 in ERY OL epithelium.

DISCUSSION
The integrity of normal stratified epithelium is maintained by a balance between proliferation of basal epithelial cells and continuous shedding of terminally differentiated epithelial cells (Jetten and Harvat, 1997; Gandarillas, 2000). Epithelial and stromal cells produce an array of cytokines and growth factors that bind to corresponding receptors on epithelial cells, which upon activation can have a wide variety of antagonistic and synergistic effects on keratinocyte proliferation and differentiation (Dotto, 1999; Friedberg et al., 2001). In this in situ study, we examined the activation status of TGF-β1 and its intracellular signaling pathway mediated through Smad molecules.

In NOM, we disclosed evidence for active nuclear Smad signaling in the epithelium: numerous Smad4 and phosphorylated Smad2/3 positive nuclei were present, with only sparse signs of

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Table I. Summary of specific stainings in NOM and ERY OL

<table>
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<tr>
<th>NOM</th>
<th>OL</th>
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<tr>
<td>LAP-TGF-β</td>
<td>Basal/parabasal layers</td>
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<td></td>
<td>Spinous/granular</td>
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<tr>
<td>LTBP-1</td>
<td>Basal/parabasal layers</td>
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<tr>
<td></td>
<td>Spinous/granular</td>
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<tr>
<td>Active TGF-β</td>
<td>Basal/parabasal layers</td>
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<tr>
<td></td>
<td>Spinous/granular</td>
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<tr>
<td>TβRI</td>
<td>Basal/parabasal layers</td>
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<tr>
<td></td>
<td>Spinous/granular</td>
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<tr>
<td>TβRII</td>
<td>Basal/parabasal layers</td>
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<tr>
<td></td>
<td>Spinous/granular</td>
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<tr>
<td>pSmad2/3</td>
<td>Basal/parabasal layers</td>
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<tr>
<td></td>
<td>Spinous/granular</td>
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<tr>
<td>Smad4</td>
<td>Basal/parabasal layers</td>
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<tr>
<td></td>
<td>Spinous/granular</td>
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<tr>
<td>Smad7</td>
<td>Basal/parabasal layers</td>
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<tr>
<td></td>
<td>Spinous/granular</td>
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<td>Smad7 mRNA</td>
<td>Basal/parabasal layers</td>
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<tr>
<td></td>
<td>Spinous/granular</td>
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<tr>
<td>PAI-1</td>
<td>Basal/parabasal layers</td>
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<td></td>
<td>Spinous/granular</td>
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</tbody>
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*0, no staining; ±, very weak staining; +, weak staining; + +, moderate staining; + + +, strong staining.

Table II. Percentages of cells with nuclei stained for Smad2/3 and Smad4 in epithelium of NOM and ERY OL (mean ± standard deviation)

<table>
<thead>
<tr>
<th></th>
<th>NOM (n=11)</th>
<th>OL (n=12)</th>
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<tbody>
<tr>
<td>pSmad2/3</td>
<td>60 ± 18</td>
<td>19 ± 11*</td>
</tr>
<tr>
<td>Smad4</td>
<td>56 ± 17</td>
<td>20 ± 12*</td>
</tr>
</tbody>
</table>

*Statistical difference between NOM and OL groups (p<0.001, t test).
Sma7 expression. This accords with observations in normal skin epidermis, where Smad2, Smad3, and Smad4 nuclear staining also was seen in the epidermal basal, spinous, and granular keratinocytes (He et al, 2001). The Smad activation indicates that this, by inference, has a physiologic regulatory function of keratinocyte proliferation and differentiation. Several factors can trigger such Smad signaling. (1) Active TGF-β1 could have been a likely activator, but this was only seen at low levels in the uppermost spinous and granular epithelial cell layers and not among basal and suprabasal keratinocytes where it should have been to exert its growth inhibitory function. This accords with observations from normal epidermis where it is also absent (Kane et al, 1991). (2) Growth factors that influence epithelial proliferation and differentiation by signaling through MAPK (e.g., epidermal growth factor and hepatocyte growth factor) can mediate activation and nuclear translocation of Smad2 (de Caestecker et al, 1998). This type of cross-talk between Smad and MAPK pathways may also exist in NOM epithelium. (3) Signaling could be induced by TGF-β2 and/or TGF-β3 (Paterson et al, 2001). This is not likely because TGF-β2 and TGF-β3 are only expressed in the upper spinous and the granular layers in NOM epithelium (own observations, data not shown) where any action on growth regulation of basal cells is unlikely. (4) Signaling could occur through activin, as activin can regulate keratinocyte differentiation (Beer et al, 2000) and mediate downstream Smad2/3-induced transcription (Lebrun et al, 1999).

In OL, the normal epithelial structure is severely disrupted, with changes such as hyperkeratosis, atrophy, and degeneration of basal cells (Andreassen, 1968; Kramer et al, 1978). In addition, the epithelium shows an abnormally high proliferation rate in the basal cell layer in the ER Y OL form of the disease (Karatsidis et al, 2003). Such changes could be caused by inhibition of the growth regulatory activity of TGF-β1. In this study we therefore examined TGF-β1 signaling in ERY OL at the level of production, activation, receptor activation and signaling pathway Smad2, Smad3, and Smad4 expression of active TGF-β1 targeted to basal keratinocytes do not survive because of a total inhibition of basal cell proliferation (Sellhuyer et al, 1993). Other transgenic strains, where latent TGF-β1 expression was coupled to keratin 14 promoter (which is conditionally activated in basal keratinocytes), showed marked defects in re-epithelialization during wound healing (Yang et al, 2001). This may be the reason why TGF-β1 during normal wound healing is activated in a confined compartment of the more superficial epithelial cell layers, at a distance from the proliferative basal cells (Kane et al, 1991). The observed restricted activation of TGF-β1 in ERY OL epithelium resembles this pattern. Our stainings for LTBP-1 and keratinocyte transglutaminase expression might give a clue to why this activation shows this confinement. In connective tissue, LTBP-1 has a function in targeting latent TGF-β to the extracellular matrix and transglutaminase contributes to this process because it cross-links LTBP-1 to extracellular matrix elements (Nunes et al, 1997). Transglutaminase is not expressed in basal keratinocytes in oral mucosa (Ta et al, 1990; present observations) and if transglutaminase and LTBP-1 have a similar TGF-β targeting function in epithelium as in connective tissue, this might explain the confinement of the TGF-β1 activation. Therefore, as long as the TGF-β1 activity remains compartmentalized as in the present ERY OL biopsies, the lesion could be regarded as a wound that is trying to heal normally but is kept in an unresolved state by the chronic inflammatory infiltrate (see below). If TGF-β1, however, would become very strongly activated and affect the basal cell layers, epithelial proliferation could become so severely inhibited that the lesion then perhaps might turn into its ulcerative form.

The epithelial differentiation pattern and cell death mode are disturbed in ERY OL (Karatsidis et al, 2003), which is the underlying cause for the morpho-pathologic changes typical for the disease. Besides inhibiting epithelial growth, TGF-β1 can affect epithelial differentiation by regulating, for example, keratin and integrin expression and apoptosis (Mansbridge and Hanawalt, 1988; Jiang et al, 1995; Zambruno et al, 1995; Min et al, 1999). Altered epidermal differentiation resembling that in OL can be seen in transgenic mice in which the TGF-β1 signaling pathway was manipulated; mice with overexpressed epidermal Smad7 show acanthosis and hyperkeratosis (He et al, 2002) and keratin-10-TGF-β1 transgenic mice in which the epidermis was treated with 12-O-tetradecanoylphorbol-13-acetate (a hyperplasia inducing agent) acquire pronounced epidermal atrophy (Cui et al, 1995). It is therefore possible that the detected inhibition of the TGF-β1 signaling pathway plays a role in the epithelial atrophy in ERY OL (Karatsidis et al, 2003).

Based on prospective follow-up studies of large groups of OLP patients, it has been suggested that OLP can be a premalignant condition because a small fraction of the initial lesions diagnosed as plaque or ERY OL over time developed into cancer (Holmstrup et al, 1988). Loss of Smad and TGF-β receptor signaling has been observed in cancer cells, and this is associated with decreased growth inhibitory control of TGF-β (Pasche, 2001). Even though the TGF-β-Smad signaling blockade was not complete in ERY OL, alterations in this growth inhibitory pathway might perhaps, in concert with other predisposing factors, play a role in dysplastic transformation of ERY OL.
Figure 1. Micrographs of immunohistologically stained tissue sections from biopsies obtained from persons with NOM (a, c, e, g, i, k, m, o, u, w) and patients with OL (b, d, f, h, j, l, n, p, r, x). After immunohistochemical staining the labeled molecules are seen in brown (counterstain with hematoxylin) and for immunofluorescence (u, v) the specific molecules are displayed in red and green (overlapping red and green appear yellow; blue nuclear stain with DAPI). Antibodies used were against (a, b) LAP-TGF-β1; (c, d) LTBP-1; (e, f) active TGF-β1 (arrows in e indicate spotted staining); (g, h) TβRI; (i, j) TβRII; (k, l) phosphorylated Smad2/3; (m, n) Smad4; (o, p) Smad7; (q, r) LTBP-1 in red, keratinocyte transglutaminase in green (position of the basement membrane indicated with white broken lines); (w, x) mature PAI-1; in situ hybridization for Smad7 (q, r, s, t; labeled molecules are in blue; counterstain with red). Low magnification pictures (lettered a to x, except for g, r, s, t) were obtained with a 20× objective (scale bar: 100 μm). High magnification pictures (without lettering) were taken with a 60× objective, except for enlargements (e) and (k), which were taken with 100× and 40× objectives, respectively. Micrographs of in situ hybridization (q, r, s, t) were obtained with a 40× objective. Frames of high magnification are connected to their respective low magnification areas with arrows. Scale bar for fluorescence figures (u, v) is 10 μm.
Figure 1. Continued
Upregulation of the expression of Smad7 can be caused by pro-inflammatory cytokines like interferon-γ and tumor necrosis factor α (Ulloa et al., 1999; Bitter et al., 2000). These are believed to be central regulatory cytokines in OL, and are synthesized and secreted by activated keratinocytes and inflammatory cells in the subepithelial infiltrate (Yamamoto and Osaki, 1995). Typically, Smad7 mRNA was observed in the basal keratinocyte layer in ERY OL. The increased basal cell proliferation in ERY OL epithelium (Karatsidis et al., 2003) can thus be due to inhibition of TGF-β/Smad signaling by Smad7, induced by interferon-γ and tumor necrosis factor α.

TGF-β is a strong upregulator of the transcription of PAI-1 in human keratinocytes (Reski-Oja and Koli, 1992). Elements in the PAI-1 promoter that are binding sites for the Smad3–Smad4 heterodimer have indeed been identified (Demnitz et al., 1998). PAI-1 expression in ERY OL epithelium was strongly reduced, which indicates a blockage at the functional level of the TGF-β/Smad signaling pathway.

In sum, these results indicate that there is a functional blockage of the TGF-β signaling pathway by increased Smad7 expression and translocation in ERY OL. This may explain the increased hyperproliferation and the disturbed terminal differentiation of keratinocytes observed in this disease.

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