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Decreased expression of p63 in oral lichen planus and graft-vs.-host disease associated with oral inflammation

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BACKGROUND: Oral lichen planus (OLP) and graft-vs.-host disease (GVHD) are conditions with increased risk of malignant transformation to squamous cell carcinoma of the head and neck (SCCHN). The *p63* gene encodes six different proteins and is expressed at high levels in SCCHN.

METHODS: Biopsies from patients diagnosed with OLP and GVHD were analysed for p63 protein expression using antibodies distinguishing between the major isoforms expressed in normal epithelia, in parallel with biopsies from normal buccal mucosa and SCCHN.

RESULTS: In OLP and GVHD a decreased expression of all p63 isoforms was seen, while expression of p53 protein was upregulated, compared with normal mucosa. In SCCHN, p63 was abundantly expressed and some tumours showed strong p53 staining, suggestive of p53 mutation.

CONCLUSIONS: Decreased p63 and increased p53 expression in OLP and GVHD indicates a coordinated action of these two related proteins to protect the oral mucosae from the damaging effects of underlying inflammation. In SCCHN disruption of the *TP53* gene and overrepresentation of certain p63 isoforms has been seen, indicating that this could lead to neoplastic transformation.

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Introduction

Oral lichen planus (OLP) is a chronic inflammatory disease of unknown origin affecting approximately 1–2% of the adult population, more frequently women than men (1.4:1) (1). Lesions are usually bilateral and mainly affect the buccal mucosa, gingiva and the lateral side of the tongue (2). The aetiology of OLP is not clear; however, an autoimmune cause has been suggested where CD8+ T cells induce apoptosis in epithelial cells within the lesion (3). OLP can associate with other autoimmune diseases such as alopecia areata, myasthenia gravis, ulcerous colitis and vitiligo (3). In OLP lesions a higher cell turnover than normal is seen (4). In contrast to lichen of the skin, OLP seems to have a chronic course with little tendency to spontaneous resolution and is highly resistant to topical treatment (5). WHO classifies OLP as a pre-malignant condition making 'mucosa more sensitive to exogenous carcinogens and thus to develop oral carcinoma' (6). Malignant transformation to squamous cell carcinoma (SCC) has been seen in 2–3% of OLP patients and females with OLP run a 50% higher risk of developing SCC of the head and neck (SCCHN) compared with a control group (6). A study comprising 1028 Swedish patients with OLP showed a statistically significant increased number of SCCHN among these patients compared with the population in general (7).

Graft-vs.-host disease (GVHD) is a serious complication after allogenic bone marrow transplantation characterized by chronic inflammation affecting skin, liver, oral mucosa and the gastrointestinal tract. Despite completely different aetiologies, oral GVHD and OLP present similar clinical appearances (8). Histopathologically, GVHD resembles OLP but has a less intense inflammatory infiltrate. In both GVHD and OLP the immune system plays a primary role in the pathogenesis, and the medical history is thus of great importance in distinguishing between the two (8). Like OLP, there are reports of development of oral SCC in areas affected by chronic GVHD (9–12).

An association between chronic inflammation and risk of developing cancer is well known (13, 14). The chronic inflammatory process provides a microenvironment, which can affect cell survival, growth, proliferation, differentiation and movement, thus contributing to cancer initiation, progression, invasion and

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metastasis (14). In addition to altered production of chemokines, cytokines and extracellular matrix components, inflammatory cells produce high levels of damaging reactive oxygen and nitrogen species, which are mutagenic and can induce cellular transformation of neighbouring cells (15). Recent studies of the cancer-prone condition, ulcerative colitis, have demonstrated the induction of a p53-mediated response to DNA damage caused by the nitrogen radical nitric oxide (NO) in non-cancerous colonic epithelia (16).

The *p63* gene is located on chromosome 3q27–29, and encodes six different proteins which are crucial for formation of the oral mucosa, salivary glands, teeth and skin (17). Each of the six different p63 proteins has different characteristics and functions, where some resemble its relative the tumour-suppressor protein p53, while others have the opposite function (18). In contrast to the p53 protein, which is expressed in all tissues, expression of p63 is limited to certain types of tissue (19, 20). Our own data indicate that the N-terminal-truncated ΔNp63, proteins are restricted to the epithelium whereas full-length, TAp63, proteins are expressed also in other tissues such as endothelium and lymphoid tissue (21, 22). The exact role of the different p63 isoforms is, however, not clear. For SCCHN, amplification of chromosome 3q21–29, which includes the p63 locus has been shown to have prognostic significance (23), and p63 is abundantly expressed in various human carcinomas, including SCCHN (21). Concerning p63 and OLP, an immunohistochemical analysis of six patients showed a significant reduction in p63 expression compared with normal oral tissue (24). Which of the p63 protein(s) that was/were suppressed in OLP was not clear, as the antibody used did not distinguish between the different p63 isoforms (24).

We have previously developed p63 isoform-specific antibodies and mapped p63 expression in normal oral mucosa as well as SCCHN (21). By analysing OLP and GVHD lesions the same way and comparing results from normal oral mucosa, SCCHN, OLP and GVHD, we wanted to map similarities/dissimilarities between OLP and GVHD lesions and normal oral mucosa as well as SCCHN.

Materials and methods

Tissue

Biopsies from 46 patients clinically diagnosed with OLP and from eight patients with clinical signs of oral manifestation of GVHD were retrieved from the archive at Clinical Pathology, Umeå University. The diagnosis of OLP was histologically verified on hematoxylin and eosin-stained slides, and all cases of OLP were in an active state of inflammation, showing a well-defined inflammatory infiltrate. None of the OLP/GVHD lesions had histologically dysplastic features. Biopsies from clinically normal buccal mucosa from 16 smokers, eight women and eight men, and 16 age- and sex-matched non-smokers and tumour tissue from 12 patients with SCCHN had previously been collected (Table 1). None of the SCCHN tumours had, to our knowledge, developed in OLP or GVHD lesions. Permission for the study was granted by the Ethical Committee (dnr 03-201; dnr 01-057; dnr 01-210).

Table 1 Data for the different groups studied: normal oral mucosa represented by smoker/non-smoker, oral lichen planus (OLP), graft-vs.-host disease (GVHD) and squamous cell carcinoma of the head and neck (SCCHN)

Diagnosis	*Sex*	*Age (range)*	*Mean age*
Smoker	8 male/8 female	26–59	45
Non-smoker	8 male/8 female	25–56	45
OLP	23 male/26 female	21–90	60
GVHD	5 male/3 female	23–61	51
SCCHN	9 male/3 female	54–82	69

Antibodies

Three antibodies specifically recognizing TAp63, ΔNp63 and p63α, respectively (21), and antibodies directed against p53 (DO7; Novocastra, Immunkemi F&D, Sweden), Ki-67 (MIB-1; Dako, Glostrup, Denmark) and CD8 (Dako) were used for staining on consecutive sections. Antibodies were used at the following concentrations: KN-TA 1:750, KN-Δ 1:2000, KN-α 1:450, DO-7 1:25, MIB-1 1:50 and CD8 1:100. As negative controls for the p63 antibodies, the corresponding pre-immune serum was used at the same concentration as the antibody. As controls for staining with monoclonal antibodies an antibody with irrelevant specificity but with the same isotype as the specific antibody was used. Sections were dewaxed, rehydrated by standard procedures and subjected to boiling in 10 mM citrate buffer (pH 6.0) for 15 min using a microwave oven for antigen retrieval. Staining was performed using a Ventana staining machine and reagents according to the supplier's recommendation. Slides were evaluated independently by two of the researchers (ME, KN) and results compared between the two. An estimation of the amount of positive cells within the whole lesion was made without specifying percentage of positivity.

Results

In evaluation of stainings, results from the two investigators (ME, KN) were concordant. In normal oral mucosa only a few cells in the basal cell layer expressed p53, whereas cases of OLP showed more p53 expressing cells. Tissue from GVHD patients showed the same pattern as tissue from OLP patients. SCCHN tumours were either completely lacking detectable p53 protein (50%) or positive for p53 (50%) at varying degrees (Fig. 1).

The Δ-2 antibody recognizes the three N-terminal-truncated p63 proteins, ΔNp63α, ΔNp63β and ΔNp63γ, and expression of these proteins has been shown to be restricted to epithelium (21). In normal oral epithelium, ΔNp63 expressing cells were detected in the basal and parabasal cell layers, and up to half or two-thirds of the epithelial thickness. In all but a few cases of OLP lesions, fewer cells expressed this subgroup of p63 and

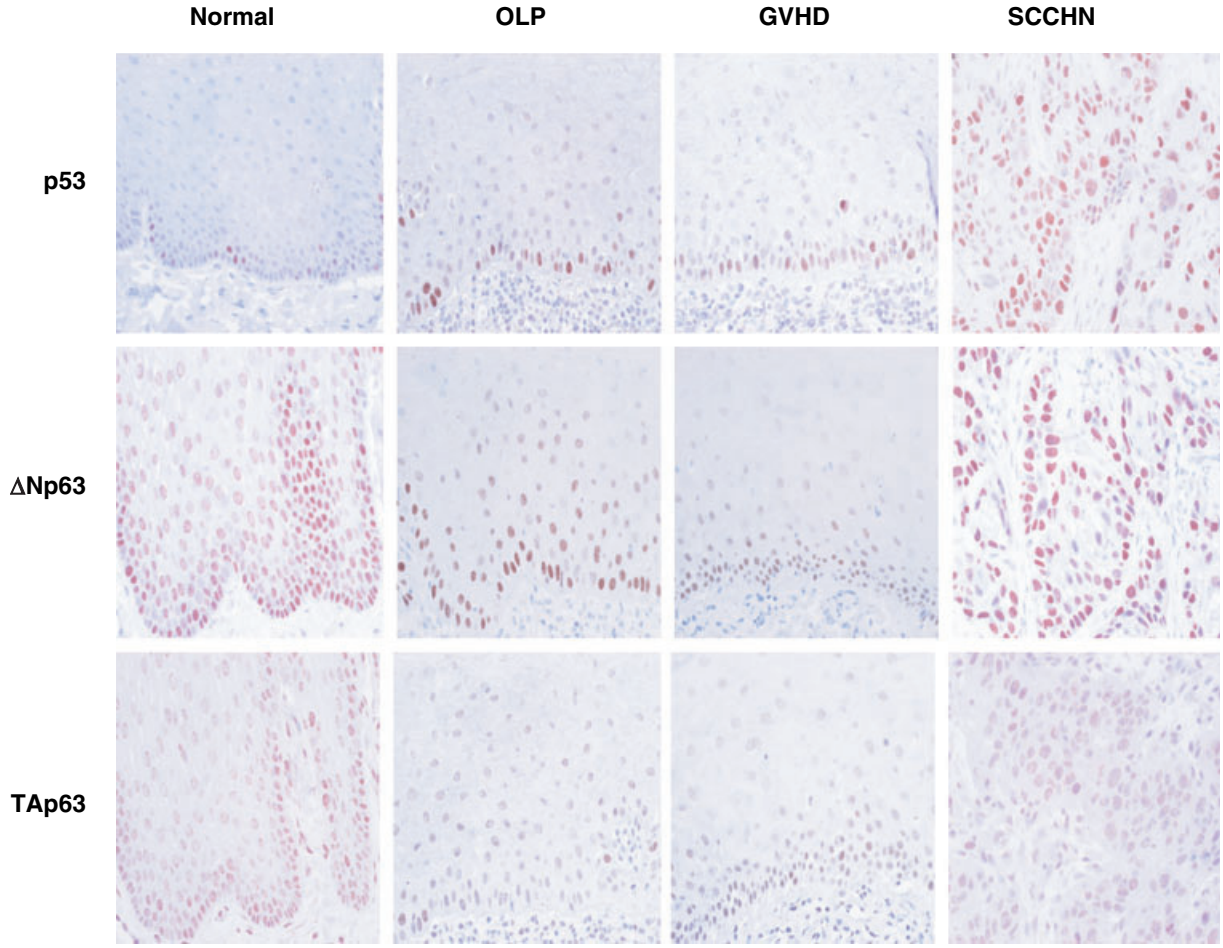

Figure 1 Immunohistochemical staining of p53 and p63 isoforms on the four groups studied: normal oral mucosa, oral lichen planus (OLP), graft-vs.-host disease (GVHD) and squamous cell carcinoma of the head and neck (SCCHN).

cells expressing these proteins seemed to have a lesser intensity. Even if immunohistochemistry is not a quantitative method, this could still indicate lower levels of ΔNp63 per cell compared with normal mucosa. The difference was particularly evident in areas with very atrophic epithelium. GVHD lesions showed the same pattern as OLP. In SCCHN, ΔNp63 proteins were detected at a similar or higher level compared with normal mucosa (Fig. 1).

In normal oral mucosa, expression of TAp63 proteins was seen all through the epithelium. Expression was in general weaker than expression of the ΔNp63 proteins. Expression of these TAp63 proteins is not restricted to epithelium, but can also be seen in endothelium and lymphoid cells. OLP lesions show the same pattern of distribution of TAp63-positive cells as normal mucosa but with lower levels of protein in individual cells. As with the ΔNp63 proteins, expression of the TAp63 proteins in GVHD lesions was in accordance with that in OLP lesions. In SCCHN, TAp63 was detected, although seemingly at slightly lower levels compared with normal mucosa (Fig. 1).

The p63α isoforms, TAp63α and ΔNp63α, in normal oral mucosa showed expression up through parts of the epithelial thickness. As with the other p63 isoforms, expression in OLP and GVHD lesions was weaker. In SCCHN expression was seen within the tumour cells (data not shown).

In comparison with OLP and GVHD lesions no obvious difference in Ki-67 expression was seen, indicating no difference in amount of cycling cells (data not shown). Cytotoxic T cells (CD8+) were mainly seen in the subepithelial inflammatory infiltrate in OLP lesions. However, a few of these cells were also seen within the epithelium, mainly in the basal half. Also in GVHD, cytotoxic T cells were seen at the same location as in OLP, although generally at lower frequency (data not shown).

Discussion

The *p63* gene, which is capable of producing six different proteins, is crucial for development of the normal oral epithelium (17). By establishing isoform-specific antibodies, we have previously seen a difference in distribution of the full-length and N-terminal-truncated p63 proteins within normal oral epithelium. In tumours derived from oral epithelium we have further seen an overrepresentation of isoforms highly expressed in the most basal cell layers (21, 22). As OLP and GVHD are

pre-malignant conditions, we were interested in determining their patterns of p63 expression compared with the patterns seen in normal mucosa and in SCCHN. All p63 isoforms studied, TAp63, ΔNp63 and p63α, showed decreased expression in OLP and GVHD lesions compared with normal oral epithelium. Compared with SCCHN, OLP and GVHD lesions had a similar decreased expression of TAp63 proteins as seen in tumour tissue, whereas expression of ΔNp63 was lower in OLP and GVHD in contrast to the increased expression of these isoforms seen in tumours (22).

The patterns of staining for p53 and p63 in mucosa from patients with OLP and GVHD are reminiscent of those seen in epithelia after induction of DNA damage following ultraviolet light exposure. UV-irradiation induces p53 stabilization, seen predominantly in basal cells, but also in parabasal cells at lower frequency (25). UV also causes a decrease in p63 expression in keratinocytes *in vitro* and in surface epithelium *in vivo* (19, 26, 27) and this decrease in ΔNp63 expression is required for UV-induced apoptosis (26). Thus, it is likely that decreased p63 and increased p53 proteins in OLP and GVHD lesions represent a protective response to increased levels of DNA damage resulting from chronic inflammation, in a manner similar to the p53 response that has been reported in other situations of chronic inflammatory damage (16). The coordinated stabilization of p53 and decreased expression of p63 enables apoptosis of epithelial cells to remove damaged cells with the potential for malignancy. In SCCHN, ΔNp63 is expressed at high levels, providing an advantage to initiated cells exposed to further damage by allowing their continued survival and thereby increasing the likelihood of accumulating the successive oncogenic alterations that are required for neoplastic transformation. Although the mechanism for induction of p63 is not clear in many cases of SCCHN, there is substantial evidence for increased expression because of gene amplification in some human tumours (28). In addition, many SCCHN contain mutated p53, further abrogating apoptotic responses and removing growth arrest pathways that operate in normal cells. On the one hand, it is possible that p53 mutations themselves arise as a consequence of increased p63 expression, but on the other hand p63 expression could increase after p53 mutations, possibly as a result of induced genomic instability in the absence of p53 function leading to *p63* gene amplification. Further studies will be required to investigate the potential role of p63 and to determine the sequence of events in which increased expression of p63 and mutation of the *TP53* gene occur during the pathogenesis of SCCHN that arise in a setting of OLP or GVHD.

In conclusion, we have used monospecific antibodies to determine the expression profiles of p63 and p53 in OLP and GVHD and identified a decreased expression of each of the p63 isoforms. The data indicate that decreased expression of p63 occurs in these pre-malignant conditions, whereas an overrepresentation of some p63 isoforms is seen in SCCHN. Additional studies into the regulation and function of p63 in oral epithelium will be useful for a fuller understanding of the mechanisms that operate within inflammatory oral lesions associated with tumour susceptibility.

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