J Oral Pathol Med (2004) 33: 264–8

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Identity of TUNEL-positive cells in the oral buccal epithelium of normal mucosa and lichen lesions

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BACKGROUND: ***In situ*** **detection of DNA fragmentation by TdT (terminal deoxynucleotidyl transferase)-mediated dUTP nick end labeling (TUNEL) is a widely used technique to identify apoptotic cells in the terminal phases of cell death. Several studies have shown that there are statistically increased numbers of TUNEL$^+$ cells within the epithelium of oral lichen (OL). It was suggested that this indicates an increased rate of apoptosis among basal and suprabasal keratinocytes in OL epithelium. The aim of this study was to identify the TUNEL$^+$ cells in the epithelium of erythematous (ERY) OL and normal oral mucosa (NOM).**
METHODS: Sections of biopsies from NOM and ERY OL were processed for TUNEL combined with immunostaining for pan-cytokeratin or for cell markers specifically expressed by different leukocytes.
RESULTS: In NOM, TUNEL$^+$ keratinocytes were almost exclusively seen in the outermost epithelial layers. This labeling was absent in ERY OL. In the basal and lower spinous layers, more TUNEL$^+$ cell nuclei were seen in ERY OL as compared with NOM, in accordance with previous studies. The present observations show, however, that only very few of these cells were keratinocytes, but rather were CD4$^+$ lymphocytes and CD68$^+$ macrophages. There was no difference between the numbers of TUNEL$^+$ keratinocytes in basal and lower spinous layers in ERY OL and NOM epithelium. No intraepithelial CD8$^+$ lymphocytes, Langerhans cells, or mast cells were found to be TUNEL$^+$.
CONCLUSION: The findings indicate that the pathologic changes in ERY OL epithelium cannot be explained by increased prevalence of terminal keratinocyte cell death identified by TUNEL.
J Oral Pathol Med (2004) **33**: 264–8

Keywords: apoptosis; epithelium; immunohistochemistry ; monocyte/macrophages; T lymphocytes

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Accepted for publication September 23, 2003

Introduction

Oral lichen (OL), comprising oral lichenoid reactions (OLR) and oral lichen planus (OLP), is a common chronic inflammatory condition that affects about 2% of the populations studied (1). OLR are typically found in contact to dental restorative materials (2). The etiologic factors behind OLP lesions are less clear, but may include stress, drug use, viral infections, and other pre-disposing factors (3). OLP and OL are indistinguishable clinically and histologically (4). Most OL lesions display a white reticular pattern, often in combination with red erythematous (ERY) and/or ulcerative areas. OL has also papular, bullous, and white plaque forms, but these are less frequent (5). Both ERY and ulcerative forms are accompanied by oral discomfort (5, 6).

The histopathology of OL is characterized by a dense inflammatory subepithelial infiltrate, predominated by T lymphocytes, and epithelial changes such as liquefaction degeneration of basal cells, atrophy, acanthosis, and hyperkeratosis (7). The ERY form is associated with epithelial atrophy, hyperproliferation, and atypical cell death (8). Numbers of TdT-mediated dUTP nick end labeled (TUNEL$^+$) cell nuclei in basal and parabasal epithelial layers have been found to be significantly higher in OL as compared with normal oral mucosa (NOM), and it has been suggested that this partly could account for pathologic changes such as degeneration of basal keratinocytes and atrophy (9–12). It has, however, not been specified whether the TUNEL$^+$ cells are keratinocytes or other cells infiltrating the epithelium. The aim of this study was therefore to identify the TUNEL$^+$ cells in the epithelium of ERY OL and NOM.

Materials and methods

Biopsy material

Permission was given by the regional Ethics Committee to conduct the investigation, and informed consent was obtained from all participants. Buccal mucosal biopsies were taken from patients with ERY OL ($n = 10$) and from healthy volunteers (NOM; $n = 7$). Parts of the ERY OL lesions also showed reticular patterns. Clinical diagnosis of ERY OL was made using the criteria described by Kramer et al. (13). After circumlesional local anesthesia with 2%

lidocaine containing 12.5 μg/ml adrenaline (Astra, Södertälje, Sweden), biopsies were excised from typically ERY areas. Absence of Candida infection in patients with ERY OL was confirmed using Dentocult® dip slides (Orion Diagnostica, Espoo, Finland).

Each biopsy was divided in two pieces for use as frozen or paraffin-imbedded samples. For frozen samples, the biopsies were snap-frozen in isopenthane on dry ice, oriented, and embedded in OCT compound and stored at −80°C. Five-micron thick sections were cut at −20°C in a cryostat, mounted on polylysine-coated glass slides (Polysine, Mezel-Gläser, Germany), and stored at −20°C until used. For paraffin samples, biopsies were fixed in 4% buffered formaldehyde solution for 24 h, processed through graded alcohols, oriented, and embedded in paraffin. Four-micron thick paraffin sections were cut, mounted on polylysine-coated glass slides, and stored at 4°C until used. Prior to staining, sections were de-waxed and rehydrated according to standard procedures. The diagnosis OL was confirmed by microscopic examination of hematoxylin-stained sections (13).

TUNEL and immunohistochemistry

Cryosections were fixed for 20 min in 4% paraformaldehyde in phosphate buffered saline (PBS). This treatment was crucial for TUNEL: without the fixation, the small DNA fragments were eluted from the sections, with no staining as a result. De-waxed and rehydrated sections from formalin-fixed biopsies could be used without any further pre-treatment. The sections were first permeabilized with a solution of 1‰ Triton® X-100 (v/v) in 1‰ sodium citrate (w/v) for 2 min at 4°C, then equilibrated in 0.5 M cacodylate buffer and incubated with TUNEL reaction mixture (2 μM biotinylated dUTP, 10 U terminal transferase, 1 mM cobalt chloride in 0.5 M cacodylate buffer) for 1 h at 37°C as described previously (14). The sections were then incubated with Cy2 (cyanine 2)-conjugated streptavidin (1 μg/ml; Amersham Pharmacia Biotech AB, Uppsala, Sweden) for 30 min followed by a nuclear staining with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI; Molecular Probes Inc., Eugene, USA) for 10 min. The TUNEL technique was verified by induction of apoptosis in murine thymi by intraperitoneal injection of dexamethasone. Major TUNEL staining was revealed as compared with untreated mice (13).

TUNEL combined with immunohistochemistry was carried out on cryosections, except for staining for cytokeratin, which was performed on paraffin sections. TUNEL was applied prior to immunohistochemistry, because antigen retrieval by heating in 10 mM citrate buffer (pH 6.0; necessary for cytokeratin staining) resulted in false positive TUNEL of nearly all epithelial cell nuclei. Antibodies against the following proteins were used: cytokeratin wide spectrum screening (pan-cytokeratin; rabbit, 14 μg/ml), CD4 (clone MT310, mouse IgG1κ, 2.3 μg/ml), CD8 (clone DK25, mouse IgG1κ, 0.4 μg/ml), CD68 (clone PG-M1, mouse IgG3, 1.9 μg/ml), and mast cell tryptase (clone AA1, mouse IgG1κ, 0.1 μg/ml) from DakoCytomation, Glostrup, Denmark. CD1a (clone 66-II-C7, mouse IgG2aκ, 10 μg/ml; Monosan, Sanbio b.v., Uden, NL, USA).

For visualization of the antibodies, sections were washed in PBS and incubated with 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 for 30 min with the appropriate secondary antibody preparation (Cy3-conjugated goat anti-mouse IgG at 4 μg/ml or Cy3-conjugated donkey anti-rabbit IgG at 2.5 μg/ml, both from Jackson ImmunoResearch Laboratories, Inc., West Grove, USA) for 30 min. This was followed by a nuclear staining with DAPI.

As negative controls, isotype-matched antibodies were used at the same concentrations as the primary antibodies, in which cases no staining was observed.

TUNEL and epithelial length measurements

Numbers of $TUNEL^+$ basal and parabasal nuclei within the entire length of each biopsy were registered. To this purpose, serial images comprising the total epithelium of each section were obtained at a magnification of 20× by means of a digital camera and monitored on a 17-in. computer screen. Measurements of the total basal cell length in each section were made by use of a software program (Soft Imaging System GmbH, Münster, Germany). The values were calculated as total $TUNEL^+$ nuclei/mm, keratin-positive $TUNEL^+$ nuclei/mm, and keratin-negative $TUNEL^+$ nuclei/mm of total measured length of the basal cell layer.

Statistics

Numbers of cells with $TUNEL^+$ nuclei in ERY OL and NOM epithelium were compared by means of two-tailed *t*-tests. Differences associated with *P*-values lower than 0.05 were considered to be statistically significant. Quantification of $TUNEL^+$ cells were made on both paraffin and frozen sections. The expression patterns and numbers of $TUNEL^+$ cells observed were similar when paraffin and frozen sections were compared (data not shown).

Results

TdT-mediated dUTP nick end labeling cells in the epithelium of ERY OL and NOM were identified by double stainings with TUNEL and specific cell markers.

In the basal and lower spinous layers, stainings with the pan-cytokeratin antibody preparation combined with TUNEL showed that very few of the $TUNEL^+$ cells in these cell layers of ERY OL and NOM were keratinocytes (Fig. 1a). There were no statistically significant differences between ERY OL as compared with NOM epithelium in the numbers of keratin-positive $TUNEL^+$ cells in basal and lower spinous layers (Table 1, quantified on paraffin sections). However, significantly increased numbers of keratin-negative $TUNEL^+$ cells were observed in the basal and lower spinous layers in ERY OL as compared with NOM epithelium (Fig. 1b; Table 1). To further identify the keratin-negative $TUNEL^+$ cells, staining for CD68 (macrophages), CD4 (T-helper lymphocytes), CD8 (cytotoxic T lymphocytes), CD1a (Langerhans cells), and mast cells tryptase (mast cells) were made in combination with TUNEL. In ERY OL, some large, round $TUNEL^+$ cells infiltrating the basal and lower spinous layers, stained for CD68 and were

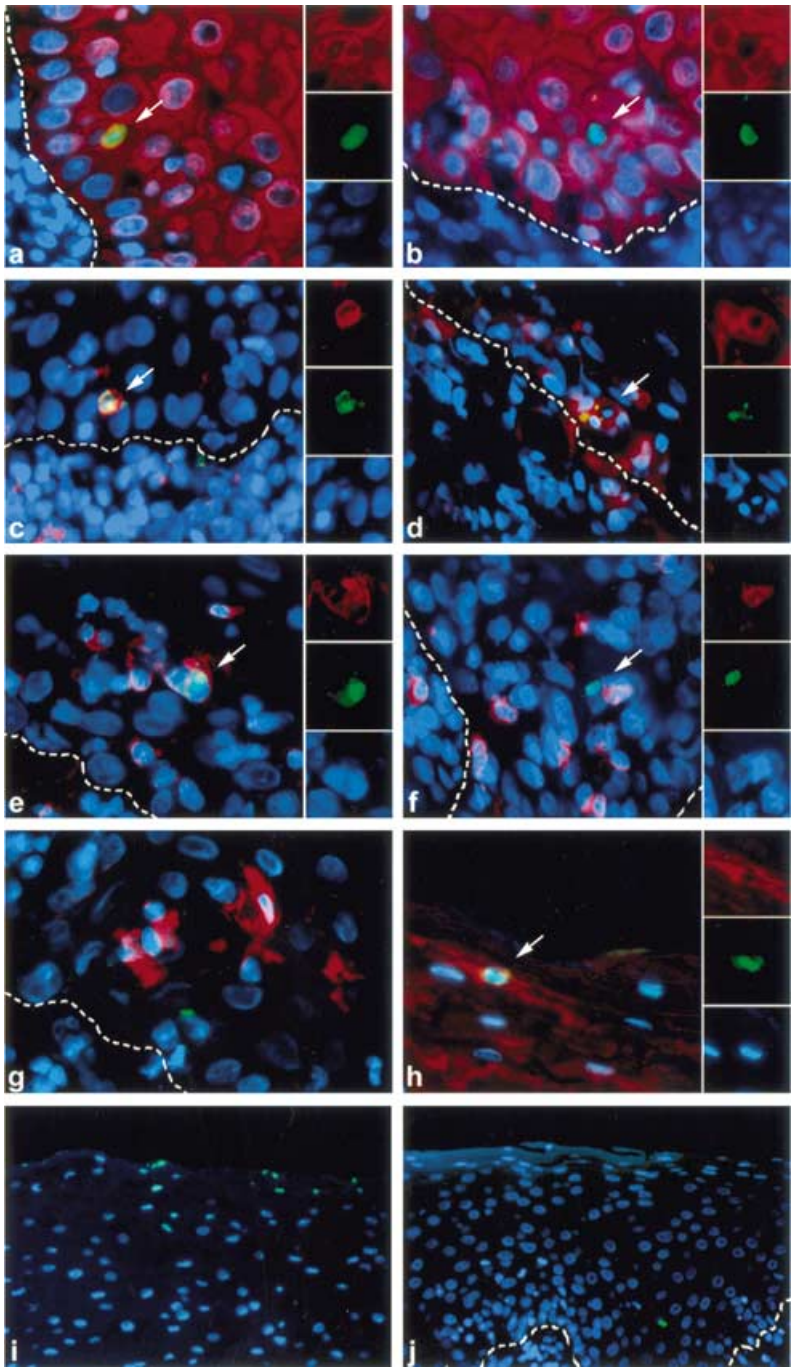

Figure 1 Micrographs of tissue sections of ERY OL (a–g,j) and NOM (h,i) stained with fluorescent reagents. TUNEL is displayed in green and other specific molecules in red (overlapping red and green appear in yellow). Cell nuclei are stained blue with DAPI. Basement membranes are indicated with white broken lines (a–g). Pictures show TUNEL together with antibodies against pan-cytokeratin (a,b,h); $CD68^+$ macrophages (c,d); $CD4^+$ lymphocytes (e); $CD8^+$ lymphocytes (f); $CD1a^+$ Langerhans cells (g). Single TUNEL is shown in pictures (i,j). Pictures (a–h) were obtained with a 100× objective, while pictures (i,j) were taken with a 20× objective. Adjacent small frames in pictures (a–f,h) show the respective single fluorescence stainings (red, green, or blue) of the areas indicated by the white arrows.

therefore identified as macrophages (Fig. 1c). Occasionally, these intraepithelial $CD68^+$ cells were seen containing $TUNEL^+$ cytoplasmic fragments, indicating that these cells might be macrophages with phagocytosed fragments from apoptotic cells (Fig. 1d). Some of the small round $CD4^+$ cells in the basal and lower spinous layers of ERY OL epithelium were also found to have $TUNEL^+$ nuclei (Fig. 1e). The very few keratin-negative $TUNEL^+$ cells in basal and lower spinous layers found in NOM epithelium were also identified as $CD68^+$ or $CD4^+$ cells. Intraepithelial $CD8^+$ lymphocytes were not found to be $TUNEL^+$, in neither ERY OL nor NOM. Strikingly, $CD8^+$ cells were

Table 1 Mean numbers (±SD) of $TUNEL^+$ nuclei/mm basal membrane in the basal and lower spinous epithelial cell layers of ERY OL and NOM biopsies

	ERY OL ($n = 10$)	*NOM* ($n = 7$)
Total $TUNEL^+$ cells/mm	$1.074 \pm 0.212^*$	0.192 ± 0.221
Keratin-negative $TUNEL^+$ cells/mm	$0.907 \pm 0.182^*$	0.049 ± 0.085
Keratin-positive $TUNEL^+$ cells/mm	0.166 ± 0.122	0.142 ± 0.188

*Statistical differences between ERY OL and NOM ($P < 0.05$; two-tailed *t*-test).

often localized in close proximity to $TUNEL^+$ cells in ERY OL epithelium (Fig. 1f). Specific staining for mast cells (mast cell tryptase) showed that these cells infiltrated the epithelium in very small numbers in the basal and spinous layers in ERY OL, but no such cells were found in NOM. Staining for CD1a showed the presence of dendritic (Langerhans) cells in both ERY OL (Fig. 1g) and NOM epithelium. Neither mast cells nor dendritic (Langerhans) cells were $TUNEL^+$.

In the superficial epithelial cell layers, NOM consistently showed a band of strongly $TUNEL^+$ nuclei in cells with a typically flattened keratinocyte morphology that also stained for keratin. (Fig. 1h,i). None of the ERY OL biopsies examined showed this feature (Fig. 1j).

Discussion

Terminal differentiation in multilayer squamous epithelium is characterized by dynamic and orderly orchestrated structural and functional changes of keratinocytes, from leaving the stem cell reservoir to their displacement and shedding at the epithelial surface. This differentiation process is crucial for the development of a protective epithelial surface layer. Prominent features of epithelial differentiation are changes in protein expression, cell morphology and intercellular adhesion, and progression towards cell death (15, 16). Various techniques can be used for *in situ* detection of different stages of cell death (17), and it has recently been questioned whether the use of TUNEL is a relevant method for identification of cell death in epidermal keratinocytes (18).

In accordance with other studies (9–12), we observed significantly higher numbers of $TUNEL^+$ cell nuclei in the basal and lower spinous layers of the epithelium in OL as compared with NOM. However, these cells were mainly identified as $CD4^+$ lymphocytes or $CD68^+$ macrophages. Very low, but similar numbers of apoptotic keratinocytes in basal and lower spinous layers were observed in ERY OL and NOM epithelium. The present study therefore contradicts the assumption that there is an increased keratinocyte cell death as identified by TUNEL in the basal and lower spinous epithelial layers in OL (9–12).

Sporadically, our stainings showed intraepithelial $TUNEL^-$ macrophages containing cytoplasmic $TUNEL^+$ fragments in ERY OL. This indicates that intraepithelial macrophages in ERY OL may phagocytose zeiotic cellular fragments derived from cells undergoing apoptosis. Macrophages that have phagocytosed apoptotic cells acquire an anti-inflammatory profile (19), and this may be one important function of these cells in OL epithelium.

The reason why certain types of leukocytes in ERY OL increasingly are apoptotic might be linked to their activation status. Macrophages and $CD4^+$ lymphocytes are important for the regulation of immunity and inflammation and increased numbers of these cell types infiltrate the epithelium in OL (20). The cells are likely to be activated because they express activation markers such as CD25 and MHC class II molecules (20). Both macrophage and CD4 T lymphocyte activation is accompanied by increased susceptibility to cell death as a part in the down-regulation of their activity (21–23). In contrast, neither intraepithelial mast cells (data not shown), $CD1a^+$ Langerhans cells, nor $CD8^+$ cells in the basal and suprabasal epithelial cell layers in ERY OL and NOM were $TUNEL^+$.

Langerhans cells' death is known to be restricted to lymph nodes (24), whereto they migrate for antigen presentation to T lymphocytes (25, 26). In contrast to $CD4^+$ cells, $CD8^+$ intraepithelial lymphocytes in NOM and ERY OL were not found to be $TUNEL^+$. Recent data suggest that the T-helper 1 lymphocyte immune profile in OL may promote $CD8^+$ cytotoxic lymphocyte activity (27). It is therefore interesting that $CD8^+$ lymphocytes in the present study readily could be observed in close proximity to $TUNEL^+$ cells: this suggests that these cells may be involved in the induction of cell death in other intraepithelial cells. This process could involve Fas/FasL (CD95/CD95L), centrally acting death-signaling molecules (21, 22). Increased expression of these molecules is indeed observed within the epithelium of OL, although without identifying the cells (9, 11).

In contrast to what was detected in the basal and lower spinous epithelial cell layers, a large proportion of keratinocytes (cells with flattened morphology and containing cytokeratin) were $TUNEL^+$ in the outermost epithelial cell layers of NOM, which is in accordance with previous observations (10, 28). This was markedly different from ERY OL because no $TUNEL^+$ cell nuclei were observed in the outermost epithelial layers in any of the ERY OL samples examined. The observations from NOM indicate that the terminal phase of cell death, as detected by extensive DNA fragmentation ($TUNEL^+$), is a late event in the normal keratinocytes' cell death pathway, and takes place just before the cells are shed from the epithelial surface. In contrast, differentiating keratinocytes in ERY OL appear to fail to reach normal maturity and become $TUNEL^+$ before shedding. Alternatively, the cells may detach from the epithelial surface concomitantly with reaching the $TUNEL^+$ stage. The concept of premature terminal differentiation in OL is further supported by the observations of early expression of differentiation markers such as involucrin and keratinocyte transglutaminase (29, 30). Interestingly, in mucosal diseases without epithelial atrophy, such as oral leukoplakia (10), oral epithelial hyperplasia and dysplasia (28), the keratinocytes in the uppermost epithelial layers become $TUNEL^+$ as is seen in NOM epithelium.

In conclusion, even though keratinocytes in ERY OL show a disturbance in their normal cell death program (decreased chromatin condensation, 8), the present results show that the majority of the $TUNEL^+$ cells in basal and

lower spinous epithelial cell layers are leukocytes and not keratinocytes. Increased terminal keratinocyte death in basal and lower spinous layers as determined by TUNEL is therefore not likely to be the cause for epithelial pathology in OL, as has been previously suggested. An aberrant and/or untimely cell death or differentiation of keratinocytes may rather cause the pathology in ERY OL epithelium.

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Acknowledgements

We thank Tannlegesenteret-Bergrådveien 13 in Oslo for help with collection of the biopsies.