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Changes in the expression of stem cell markers in oral lichen planus and hyperkeratotic lesions

Osman Köse[1], Anand Lalli[1], Adegun O. Kutulola[1], Edward W. Odell[2] and Ahmad Waseem[1]

[1]Programme in Muco-Cutaneous Oncology, Department of Clinical and Diagnostic Oral Sciences, Barts' and The London NHS Trust, Queen Mary's School of Medicine and Dentistry, Institute of Cell and Molecular Science, London, UK
[2]Department of Oral Pathology, King's College London, London, UK

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Abstract: Despite the pivotal role of stem cells in homeostasis of oral epithelia the location of this cell population within the tissue is uncertain. How disease influences these cells *in vivo* also remains to be elucidated. In this study we have used six molecular markers to identify stem cells in normal and diseased buccal mucosa. Samples of normal oral mucosa (NOM), hyperkeratosis (OHK) and oral lichen planus (OLP) were immunostained for α6 and β1 integrins, keratin 15 (K15), melanoma-associated chondroitin sulphate proteoglycan (MCSP), NG2 the rat homologue of human MCSP and notch 1. K15, NG2 and β1 staining was continuous in the basal layer of NOM whilst α6 and MCSP were limited to basal cells at the tips of connective tissue papillae. K15 was downregulated in OLP whereas α6, β1 and MCSP were upregulated in both OLP and OHK. NG2 remained unchanged and notch 1 was absent in all samples. Therefore, the stem cell phenotype in OLP and OHK maybe altered in response to pathological signaling. Classification of these changes is essential to understand the role of adult stem cells in the pathogenesis of oral diseases characterised by abnormal keratinocyte proliferation and differentiation. (J. Oral Sci. 49, 133-139, 2007)

Correspondence to Dr. Ahmad Waseem, Department of Clinical and Diagnostic Oral Sciences, 4 Newark Street, London E1 2AT, UK
Tel: +44-207-8822387
Fax: +44-207-8827153
E-mail: a.waseem@qmul.ac.uk
Present address for Dr. Osman Köse: Department of Dermatology, Gulhane School of Medicine (GATA), Ankara, Turkey

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Introduction

Oral epithelium is formed of stratified layers of keratinocytes that serve predominantly as a protective barrier against the external environment. The proliferation of keratinocytes is confined to the basal layer adjacent to the basement membrane whereas differentiation occurs in the suprabasal layers. Keratinocytes undergo a process of sequential terminal differentiation in each successive suprabasal layer from the basement membrane and are constantly shed at the mucosal surface during function, therefore proliferation is continually required to maintain structural integrity. The self-renewing ability of the epithelium is bestowed by adult stem cells that are found in the basal layer and play a vital role in epithelial homeostasis throughout the lifetime of the organism.

Stem cells possess unlimited self-renewal capacity but rarely undergo cell division (1). A stem cell can either divide symmetrically to produce two identical stem cells or divide asymmetrically to regenerate and produce a differentiated transit-amplifying (TA) cell (reviewed in (2)). The TA cells migrate laterally to populate the basal layer and undergo limited rounds of rapid cell division to increase the TA pool of cells, which then become committed to undergo terminal differentiation in the suprabasal layers. This mechanism allows a large number of TA cells to be produced from a single stem cell division thus protecting the stem cells from acquiring genetic lesions and consequent potential neoplastic change, as DNA is most at risk from

environmental mutagenic agents whilst cells are actively dividing (3).

Despite the pivotal role of stem cells in the maintenance of epithelial structural integrity there remains confusion about how to identify them *in vivo*. The best method is based on the quiescent property of stem cells, which incorporate and retain nucleotide analogue labels in their DNA for much longer than their more mitotically active progeny (4). However, this approach cannot ethically be applied to humans therefore molecular markers which specifically identify the adult stem cell phenotype are required. The bulge region of the human hair follicle is an undisputed site of label retaining adult stem cells in the epidermis (5) and molecular characterisation of this cell population has defined a series of stem cell markers in the encoded mRNA (6). However, the hair follicle stem cells may be distinct from their interfollicular basal layer counterparts because they are required to produce all the different cell types of the hair follicle and not just replenish a population of keratinocytes. In addition, it is well accepted that there is poor correlation between cellular mRNA and functional protein levels (7,8). Nevertheless, some of these markers of hair follicle stem cells have also been suggested as indicators of stem cells in interfollicular epidermis but their relevance to the structurally similar oral epithelium is unknown.

In this study we show the location of stem cells in normal non-keratinised buccal epithelium (NOM) by immunohistochemical staining for the putative stem cell markers α6 and β1 integrins, melanoma-associated chondroitin sulphate proteoglycan (MCSP), NG2 the rat homologue of human MCSP, notch 1 and keratin 15 (K15). This is the first study to show alterations in stem cell marker expression in oral lichen planus and oral hyperkeratotic lesions which indicates pathological signalling may regulate expression of these markers. This implicates adult stem cells in the pathogenesis of these mucosal disorders where epithelial differentiation and proliferation is known to be perturbed.

Materials and Methods

Antibodies and tissue samples

The mouse monoclonal antibody Ab-1 (clone A6) against human notch 1 was commercially obtained (Lab Vision, Fremont, USA) as were 4F10 against α6 integrin (AbD Serotec, Oxford, UK) and N143.8 against NG2 neuronal chondroitin sulphate protein (Invitrogen, California, USA). The remaining antibodies were raised in-house and were obtained by growing the respective hybridomas in DMEM plus 10% foetal calf serum. The confluent cultures were spun and the supernatants stored in 0.02% (w/v) sodium azide at 4°C until used. The hybridoma clones used for antibody production were: LHM2, against MCSP core protein (9), LHK15, against K15 (10), and P5D2 against β1 integrin (11).

Samples of site matched normal (3 patients) and diseased mucosa; hyperkeratotic lesions (6 patients) and lichen planus (4 patients) were collected for diagnostic purposes with ethical committee approval for inclusion in this study. Samples were snap frozen and mounted in Cryo-M-Bed (Bright Instruments, Cambridge, UK) on cork discs. 5μm sections were cut in a cryostat (Bright Instruments, Cambridge, UK) and thaw mounted onto Superfrost + slides (Menzel-Glaser, Brunswick, Germany) and stored at -80°C until required.

Immunhistochemistry

For immunohistochemical staining slides were defrosted and dried at room temperature for 1h before fixing in methanol:acetone (1:1) at 4°C for 10 min and treated with hydrogen peroxidase (3%) for 2 min. Sections were then blocked with 50% (v/v) horse serum in PBSA for 5 min before being incubated with 10-20μg/ml of primary antibody in a humid chamber at room temperature for 90 min and then biotinylated rabbit anti-mouse secondary antibody (1:300) (DAKO, Cambridge, UK) for 30 min. The sections were then treated with streptavidin-horseradish peroxidase (1:50) (DAKO, UK) for 30 min and colour developed for 1 min in 200μg/ml 3,3'diaminobenzidine (Vector Labs, Peterborough, UK) before light counterstaining in haematoxylin and mounting in DP mountant (Merck, West Drayton, UK). Negative controls were performed by incubating in PBSA alone. Sections were observed and photographed on a Microphot FXA microscope (Nikon, Tokyo, Japan) and Coolpix 990 camera (Nikon, Japan) and the photographs assembled using Adobe Photoshop CS2.

Results

In this study we have shown, by immunohistochemistry, the expression of six putative stem cell markers (K15, β1 integrin, α6 integrin, NG2, notch 1 and MCSP) in normal non-keratinised buccal epithelium (NOM), oral hyperkeratotic lesions (OHK) and oral lichen planus (OLP) (summarised in Table 1).

In NOM expression of K15 and NG2 was uniform throughout the basal layer of the epithelium in all samples (Figs. 1a and 1j). MCSP staining was only observed in small colonies of basal keratinocytes located at the tips of the connective tissue papillae (Fig. 1g). α6 integrin displayed similar localisation but there was additional epibasal staining at the tips of the connective tissue papillae and

Table 1 Expression of stem cell markers in samples of normal oral mucosa, oral hyperkeratotic lesions and oral lichen planus

Sample	K15	β1 integrin	α6 integrin	MCSP	NG2	Notch 1
NOM#	B (+++)	B (++)	B, SB (+)	B (+)	B (+)	-
OHK1	B (+++)	B (+++), SB (+)	B, SB (+)	B (+)	B (+)	-
OHK2	B (+++)	B (+++), SB (+)	B (++), SB (+)	B (++)	B (+)	-
OHK3	-	B (++), SB (+)	B (++), SB (+)	B (+)	B (+)	-
OHK4	B (+++)	B (+++), SB (+)	B, SB (+)	B (+)	B (+)	-
OHK5	-	B (+++), SB (+)	B (++), SB (+)	B (++)	B (+)	-
OHK6	-	B (++), SB (+)	B (++), SB (+)	B (+)	B (+)	-
OLP1	-	B (+++), SB (++)	B (++), SB(+)	B (++)	B (+)	-
OLP2	-	B (+++), SB (++)	B (++)	B (++)	B (++)	-
OLP3	-	B, SB (++)	B (++), SB (+)	B (+++)	B (+)	-
OLP4	-	B, SB (++)	B (++), SB (+)	B (++)	B (+)	-

NOM normal oral mucosa; OHK oral hyperkeratotic lesions; OLP oral lichen planus; B basal, SB suprabasal; (-) no staining, (+) weak, (++) moderate and (+++) strong staining.
#The expression in NOM was an average of three samples.

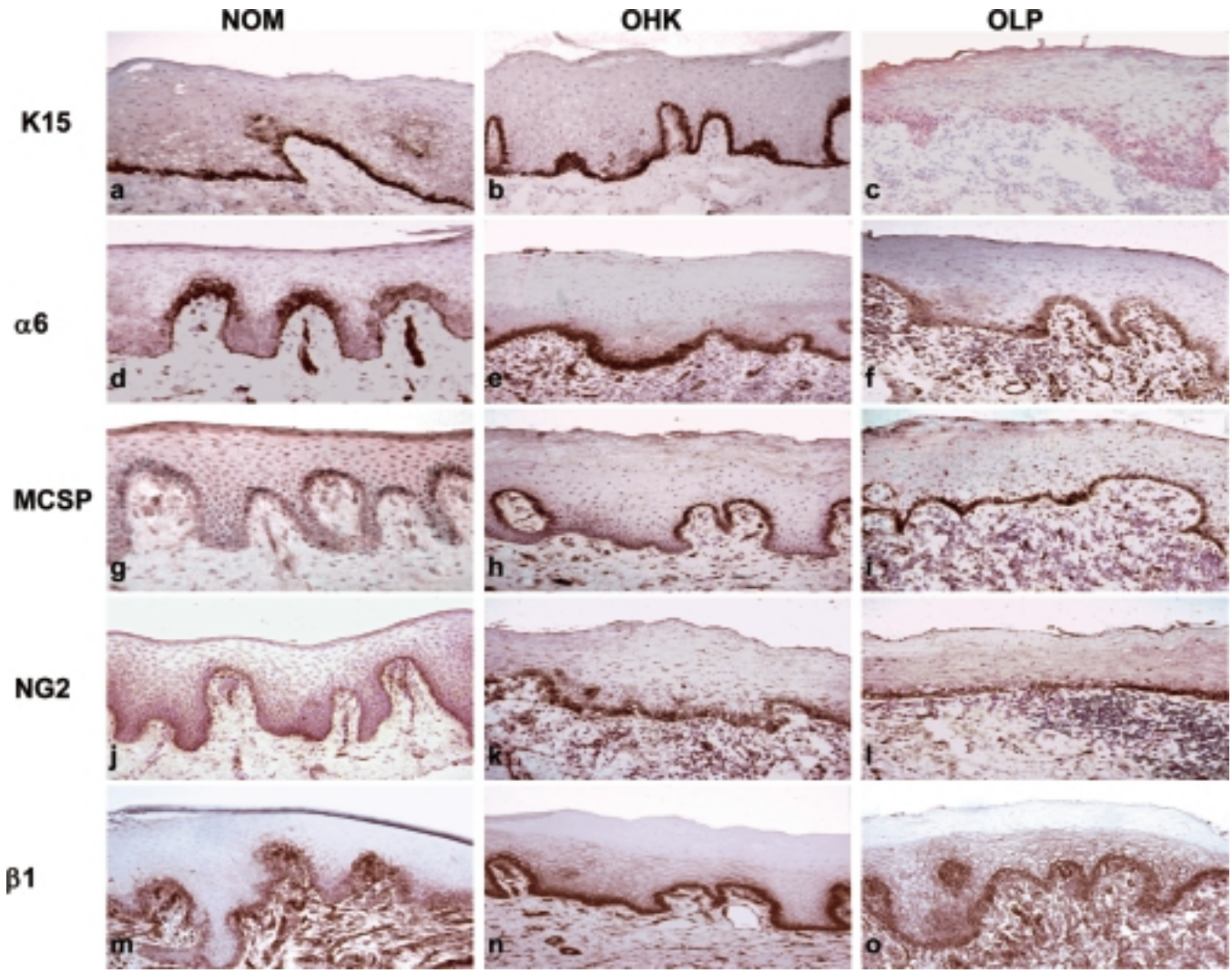

Fig. 1 Immunohistochemical staining for the putative stem cell markers: keratin 15 (K15); α6 integrin; β1 integrin; melanoma-associated chondroitin sulphate proteoglycan (MCSP) and NG2 the rat homologue of human MCSP, in normal oral mucosa (NOM) and the mucosal diseases; oral lichen planus (OLP) and oral hyperkeratosis (OHK). Original magnification ×100

overall a larger proportion of the basal cells were positively stained than for MCSP (Fig. 1d). β1 expression was continuous throughout the basal layer but also showed some expression in the epibasal cells at the tips of the connective tissue papillae (Fig. 1m). No samples stained positive for notch 1 (not shown).

In 50% of OHK samples expression of K15 was absent whilst in the remainder K15 staining was uniformly strong in the basal layer (Fig. 1b). β1 integrin displayed a similar staining pattern to the K15 positive OHK samples and staining intensity was qualitatively stronger than for β1 in NOM (Fig. 1n). α6 integrin expression was substantially different from NOM with uniform staining throughout the basal and epibasal layers (Fig. 1e) contrasting with the interrupted pattern seen in NOM. MCSP staining was comparable to NOM as expression was limited to the tip of the connective tissue papillae (Fig. 1h). As in NOM staining for NG2 was uniformly basal (Fig. 1k) and all samples were notch 1 negative.

Amongst the four samples of OLP analysed none expressed K15 to a level detectable by immunohistochemistry (Fig. 1c). Other putative stem cell markers also showed significant variation compared with NOM. β1 staining was continuous in the basal layer of OLP samples and stronger expression was observed in the suprabasal layers (Fig. 1o). MCSP also displayed continuous expression in the basal layer (Fig. 1i) contrasting with the interrupted staining seen in both NOM and OHK. NG2 staining remained solely basal (Fig. 1l) whilst α6 expression mimicked that in OHK with uniform basal and epibasal staining (Fig. 1f) distinct from NOM where only the tips of the connective tissue papillae stained positive. As in NOM and OHK, notch 1 expression was absent in OLP.

Discussion

The stem cell markers we observed in normal buccal epithelium have been reported to identify adult stem cells in a variety of tissues *in vivo*, including interfollicular epidermis which can be regarded as structurally and functionally comparable to oral epithelium. In our study K15, NG2 and β1 integrin showed continuous basal staining which could indicate a greater number of stem cells in the basal layer reflecting the greater proliferation rate, reportedly twice that of the epidermis, required to maintain the functional integrity of oral epithelia (12). However, it is estimated that only 1% to 10% of all basal cells in the epidermis are stem cells (13) implying that these markers may not be specific for the stem cell population in oral epithelium. The staining observed for MCSP and α6 integrin was restricted to basal cells at the tips of the connective tissue papillae which suggests that the stem cell population is localised to this region and is consistent with the model presented by Jensen and co-workers (14) based on human thin interfollicular epidermis. However, the difficulties of accurately interpreting 3D tissue structure from 2D sections are well recognised (15). For example, specific staining for a basal layer antigen may appear in isolated patches of suprabasal keratinocytes due to misalignment between the plane of the basal lamina and the plane of the section (16) which results in difficulties in the interpretation of basal and epibasal discontinuous staining, such as that with α6 integrin in NOM (Fig. 1d). Alternative models for the organisation of the proliferation and differentiation compartments in NOM do not correlate with the staining patterns observed in this study. These include the epidermal proliferative unit (EPU) model derived from thin murine interfollicular epidermis (17), where stem cells in the basal layer are responsible for repopulating discrete hexagonal columns of epidermis producing a single cornified squame, to be shed at the tissue surface. The remaining possibility is adult stem cells deeply located, either at the tips of rete-ridges as has been proposed for thick palmar and plantar epidermis (18) and the keratinised mucosa of the palate (19) or in the interpapillary basal layer of oesophageal mucosa (20), where these cells' DNA is most protected from environmental mutagens. Therefore, it appears that the position of the stem cell compartment within a tissue varies at different anatomical sites which may be related to the functional role or embryological origin of the epithelium and be mediated by the composition of the underlying extracellular matrix (ECM) at the anatomical site (20).

From the staining patterns observed, in our study, it appears that α6 integrin and MCSP are the most likely adult stem cell markers in oral epithelium, due to their restricted expression in the basal cell population. High expression of the integrin family of receptors is believed to be responsible for stem cells being more adhesive to basement membrane than other basal cells (21). Basal keratinocytes predominantly express α2β1, α3β1 and α6β4 integrins which are known to regulate cell-cell and cell-ECM adhesion and commitment to terminal differentiation (22,23). High expression of α6 and β1 integrin subunits have been shown to be key markers of stem cell characteristic in the human epidermis (21,24). However, evidence from human epidermal studies also indicates that it may require a number of markers to resolve candidate stem cells from TA cells in the basal layer. For example, expression of high levels of α6 integrin in combination with low levels of another cell surface receptor (CD71) more accurately indicates the epidermal stem cell phenotype (25).

MCSP is also reportedly a stem cell marker in human epidermis where it is co-expressed with β1 integrin, discontinuously within the basal layer, at the tips of dermal papillae (9). This proteoglycan is believed to be important in regulating adhesion during epidermal keratinocyte differentiation and MCSP is known to stimulate α4β1 integrin mediated cell adhesion and spreading (26), however, despite this specific integrin not being expressed in human keratinocytes the mechanism may still be applicable (27). NG2 is a membrane associated chondroitin sulphate proteoglycan with significant gene-sequence homology to MCSP. NG2 is a marker of mitotically active stem cell progeny in the human central nervous system (28) whereas MCSP indicates mitotically inactive epidermal stem cells. This corresponds well with our finding of more NG2 positive cells within the basal layer, indicating a larger pool of TA cells and fewer MCSP positive cells indicating a smaller number of adult stem cells. Interestingly the expression pattern of NG2 is identical to MCSP in epidermis which may indicate that the distribution of proteoglycan antigen in oral epithelial adult stem cells is different from those in interfollicular epidermis. However, it remains to be seen whether the NG2 antibody used here cross reacts with other antigens specific to oral basal keratinocytes.

MCSP is believed to be involved in stem cell clustering within the epidermis which produces niches within the basal layer conducive to maintaining the stem cell phenotype (29). This is represented by the expression pattern seen in NOM where staining is limited to a few adjacent basal cells at the tips of the connective tissue papillae. As well as adhering to the basement membrane stem cells are believed to be less mobile than other basal cells therefore it was surprising to find no evidence of notch 1 expression as the notch pathway is known to decrease keratinocyte motility (30) in addition to its important role in controlling the fate of undifferentiated cell populations (31). Notch 1 expression has been shown in the epibasal stratum spinosum of stratified squamous cervical epithelium but is not present in the basal layer or in simple columnar endocervical epithelium, implying expression maybe restricted to complex epithelium where it defines those cells exiting proliferation and entering a differentiation stream (32).

Stem cells must play a role in many mucosal disorders characterised by abnormal proliferation and differentiation of keratinocytes, such as OLP, OHK, dysplasia and carcinoma. Lichen planus is a common chronic inflammatory disease of unknown aetiology affecting up to 2% of the population. Histologically, OLP is characterised by T lymphocyte-mediated degeneration of the basal layer keratinocytes, either by direct cytotoxicity or the effect of an altered cytokine environment. Changes in markers of basal cell phenotype, such as reduced expression of K5 and K14, have been reported previously and attributed to basal cell degeneration (33). Therefore, the complete loss of basal K15 expression, observed in our samples, may be symptomatic of the same pathological process. The other changes seen in OLP, such as increased α6 and β1 integrin and MCSP expression, were also present in OHK which is a different pathological process resulting in increased cell turnover and altered terminal differentiation without basal cell damage. OHK lesions are histologically characterised by a thick layer of ortho- or para-keratinised oral epithelium and therefore present clinically as white patches on the oral mucosa. Increased staining could be the result of more stem cells due to a switch by the stem cell population to favour symmetric division in response to pathological signalling. However, this seems unlikely as the histology indicates there must be increased TA cell production to maintain the diseased epithelium. Alternative explanations include re-expression of these markers by stem cell progeny as a result of altered signalling or that some expression is retained by early TA cells. The latter could be a normal pattern of expression that is not evident, by immunohistochemistry, in the stable state because of the small number of first division daughter cells from asymmetric stem cell division. Either explanation could be consistent with the increase in keratinocyte proliferation observed in OLP and to a lesser extent OHK (34). This is also shown by MCSP expression where the number of positive basal cells in OLP is greater than in OHK or NOM, though the very variable reactive proliferation in OLP makes interpretation difficult. Staining for α6 and β1 integrin was also more evident in OLP and OHK than NOM, which has previously been reported to correlate with keratinocyte proliferation in these mucosal diseases (35,36).

In conclusion, we have described the expression of six putative stem cell markers in normal buccal epithelium and shown their expression to be altered in the oral mucosal diseases, OLP and OHK. This suggests that stem cell marker expression maybe altered by pathological signalling in these lesions. Further studies of these molecular perturbations are essential to understand the fundamental role of adult stem cells in the pathogenesis of benign mucosal disease.

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