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The immunohistology of CD40 in human oral epithelium in health and disease

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BACKGROUND: CD40 has a role in the regulation of immune responses, cell proliferation and migration, and apoptosis. Little is known of its distribution in oral mucosal pathology.

METHODS: Oral keratinocyte lines were tested for CD40 protein by Western blotting. Immunohistochemistry was used to stain paraffin sections of oral mucosa in health and in inflammatory, reactive, dysplastic and malignant disease.

RESULTS: Western blotting confirmed the presence of CD40 in oral keratinocytes. CD40 was generally expressed by keratinocytes in the basal layer, with variable parabasal expression. Langerhans cells also stained positively. Expression was lost in nine of 33 (27%) epithelial dysplasias, seven of which were severe. Eighty-one percent of well, 69% of moderately and 50% of poorly differentiated oral squamous cell carcinomas (OSCC) expressed CD40. Overall, 45 of 65 (69%) OSCC were positive. The pattern of expression was unrelated to tumour differentiation.

CONCLUSION: CD40 expression by basal and parabasal oral keratinocytes is physiological. Expression is lost in approximately one-third of oral epithelial dysplasias and OSCC. The significance of such loss remains unknown, but may be related to immunological or other abnormalities of keratinocyte homeostasis.

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Introduction

CD40 and its ligand CD40L (CD154) are phosphorylated glycoproteins of 48 and 33 kDa respectively, belonging to the tumour necrosis factor receptor superfamily. CD40 is composed of a 171 amino acid extracellular domain, a 22 amino acid transmembrane domain, and a 62 amino acid cytoplasmic tail (1, 2). Five CD40 isoforms (numbered I–V) have been identified (3). CD40 is expressed by a diverse range of cell types, reflecting its importance in a number of biological functions. It is an essential immune co-stimulatory molecule, and CD40–CD40L interactions have a central role in both cellular and humoral immune responses (4, 5). Ligation of CD40 also plays a part in the regulation of apoptosis, though the mechanisms that control CD40 modulation of inhibition (6) or activation (7) of apoptosis remain unclear. In normal epidermis and the stratified squamous epithelium covering oral mucosa, the expression of CD40 is restricted to keratinocytes and Langerhans cells of the basal and parabasal layers (8–14), consistent with a function related to epithelial proliferation and immunological potency. We have recently reported *in vitro* data, which support this. Transfection and ligation of CD40 into a CD40-negative human oral keratinocyte line inhibited or promoted cell proliferation (depending on which ligand was bound), promoted migration, but did not effect apoptosis *in vitro* (15). Transfection of CD40 into the same CD40-negative oral keratinocyte line upregulated the ability to induce T lymphocyte proliferation and induced expression of other immunologically potent costimulatory molecules such as CD80 and CD86 (16). This is consistent with an immune function for oral keratinocytes in pathological situations, when they express class II molecules of the major histocompatibility complex (13, 17–21). Most of the available data suggest that oral keratinocytes are ubiquitously CD40-positive *in vitro* (11–13, 22) and in oral squamous cell carcinoma (OSCC) (11, 12, 14). However, the identification of a cell line derived from OSCC, which is CD40-negative, raises the question of how frequently this occurs *in vivo*. To date, only 35 OSCC have been tested for CD40 expression (11, 12, 14). Inflammatory and potentially malignant oral diseases have yet to be investigated in this respect. We wished to correlate our *in vitro* data (15, 16) with the histological distribution of CD40 in oral disease. Firstly, we aimed to demonstrate CD40 protein in well-characterised keratinocyte lines derived from OSCC. Secondly, using

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immunohistochemistry in tissue sections, we aimed to determine the expression and cellular distribution of CD40 in the epithelium of normal oral mucosa, and in inflammatory, dysplastic and malignant oral epithelial lesions.

Methods

Cell lines and Western blotting

The human keratinocyte lines H357, H376, H157, H103, H314, H413 and H400, all derived from OSCC (23), were cultured in keratinocyte growth medium (24). The Epstein–Barr virus-transformed B lymphocyte line FC-7 (25) acted as a positive control.

When confluent, the keratinocytes were covered with 150 µl of ice-cold RIPA buffer, containing 1% Triton X100, 0.5% deoxycholate (Sigma, Gillingham, UK), 0.1% sodium dodecyl sulphate (SDS), 1 mM Na_3VO_4 (Sigma) and 5% freshly added protease inhibitor cocktail (Sigma) and the flasks placed on ice for 30–60 min. The cells were then scraped into microfuge tubes and centrifuged at 230 *g* for 10 min. The supernatant was transferred into fresh tubes and stored at −20°C until required. A measure of 10^6 FC-7 cells were washed with phosphate buffered saline (PBS), centrifuged at 230 *g* and 100 µl of ice cold RIPA buffer with protease inhibitors added to the pellet. Tubes were placed on ice and a lysate was prepared in the same manner as described above. A colorimetric assay was used to determine the protein concentration within each cell sample according to the manufacturer's instructions (Bio-Rad, Hemel Hempstead, UK). Twenty micrograms of extracted protein was separated using a 10% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane (Amersham Biosciences, Chalfont St Giles, Bucks, UK). Membranes were placed in blocking solution [5% dried milk in PBS with 0.05% Tween 20 (Sigma) (PBST)] for 1 h at room temperature, washed thrice for 10 min each in PBST followed by incubation with polyclonal rabbit anti-CD40 primary antibody (1/200 dilution) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) (26) in PBST, at room temperature for 1 h with constant agitation. The membrane was rinsed thrice and then incubated in horseradish peroxidase-labelled secondary antibody (1/50) (Dako, Ely, UK) diluted in PBST for 30 min. The wash step was repeated and bound antibody was visualised by enhanced chemiluminescence and autoradiography (Amersham).

Tissues

Ethical approval was sought and granted to stain paraffin sections of normal oral mucosa (NOM) ($n = 10$), inflammatory and reactive oral mucosal conditions ($n = 57$: 24 non-specific hyperkeratoses, 21 lichenoid reactions, seven fibro-epithelial polyps, four non-specific inflammation/ulceration and three viral papillomas), oral epithelial dysplasias ($n = 33$; seven mild, eight moderate and 18 severe) and OSCC ($n = 65$, 53 males, 12 females, mean age 59.23 ± 1.54 years). Cervical lymph nodes retrieved during neck dissections were used as positive controls.

Immunohistochemistry

Six-micrometer-thick sections were immersed in 600 ml of Target Retrieval Fluid (Dako) (pH 6.0, not topped up during the procedure) and microwaved at full power (approximately 850 W) from cold for 25 min and incubated in 1/100 mouse anti-human CD40 monoclonal antibody (mAB89, Immunotech, Beckman Coulter UK Ltd, High Wycombe, Bucks, UK) (27). Serial sections of 20 cases (eight OSCC, one severe dysplasia, five hyperkeratoses, one fibro-epithelial polyp, two lichenoid reactions, three papillomas) were also stained with 1/100 mouse anti-human CD1a monoclonal antibody (010, Immunotech) (28). Antigen-binding sites were visualised with the EnVision+ peroxidase system (29). Controls included replacement of the primary antibody with normal swine serum, non-immune mouse serum and an 'irrelevant' primary antibody of identical isotype for each primary antibody. Sections of cervical lymph nodes acted as positive controls. All sections were analysed with a Zeiss Axioplan 2 microscope and photographed using an AxioCam HRc digital camera. Staining was deemed to be positive if it was as intense as that seen in lymph node controls. Follow-up clinical data on OSCC were inadequate for statistical analysis, which was therefore not carried out.

Results

Western blotting

In lysates from all keratinocyte lines, anti-CD40 antibody reacted with a band corresponding to approximately 48 kDa (Fig. 1).

Normal oral mucosa

The general pattern was of membranous and cytoplasmic expression by keratinocytes, either focal or diffuse, in the basal layer (Fig. 2a). There was intra-individual variation in the pattern of staining (Figs. 2b and c). Membrane expression extended into the immediate parabasal stratum spinosum to variable degrees. Scattered suprabasal cells with a dendritic morphology also stained positively. Keratinocytes in the most superficial strata were negative. Fibroblasts and endothelial cells in the lamina propria were strongly positive.

Inflammatory and reactive disease

Suprabasal membrane expression was more marked where there was chronic inflammation (Fig. 3a). The only exceptions to this were absence of keratinocyte expression in one lichenoid reaction. Serial sections stained for CD1a indicated that the suprabasal dendritic

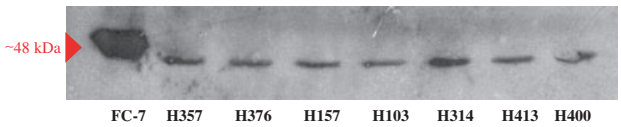

Figure 1 Western blot analysis of CD40 expression by keratinocyte lines. Fifteen micrograms of protein were loaded in each lane under non-reducing conditions. The FC-7 cell line was used as a positive control (first lane).

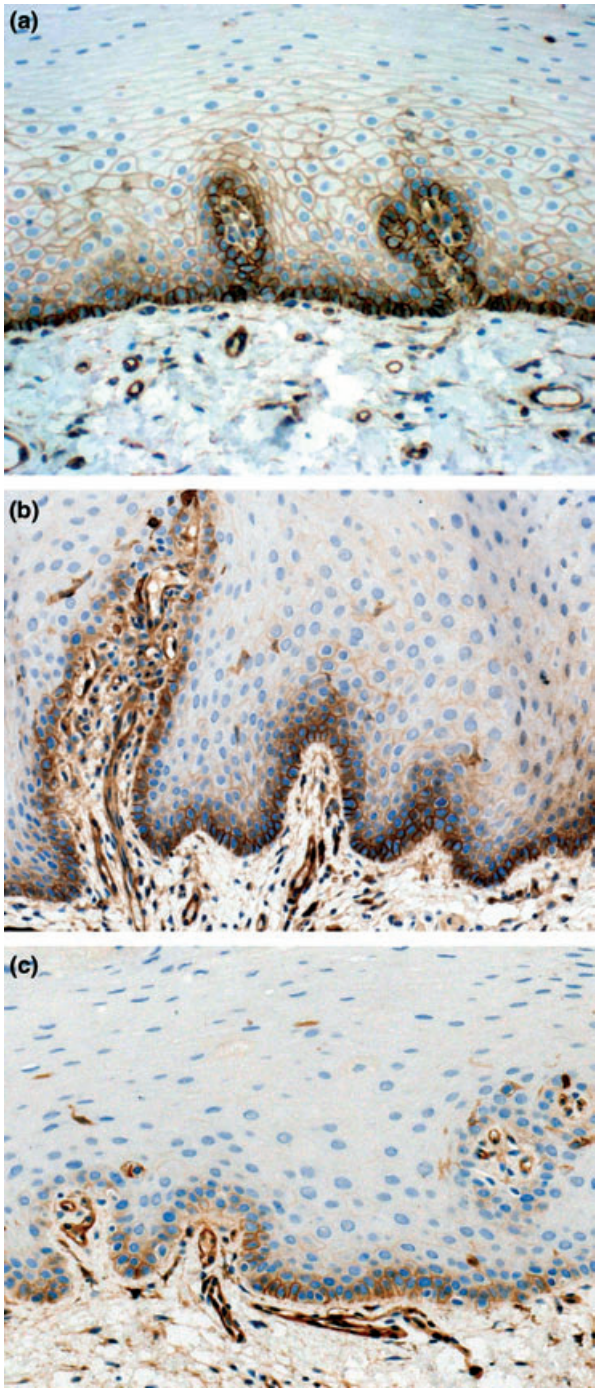

Figure 2 Immunohistochemistry of CD40 in epithelium covering normal oral mucosa. (a) There is strong membrane and cytoplasmic expression in the stratum basale and a reticular pattern of membrane staining in the stratum spinosum. Few dendritic cells are seen; (b) CD40 is expressed by basal keratinocytes and dendritic cells within the epithelium. The suprabasal reticular staining is much weaker than in (a); (c) different field from same section as (b). Basal staining is less intense than in (b) and there is minimal suprabasal staining. In all fields, endothelial cells and spindled fibroblasts within the lamina propria are also positive. Field width: 450 μm.

cells were Langerhans cells (Fig. 3b). Subjectively, the density of CD1a-positive dendritic cells was comparable to (10 cases), greater than (eight) and less than (two)

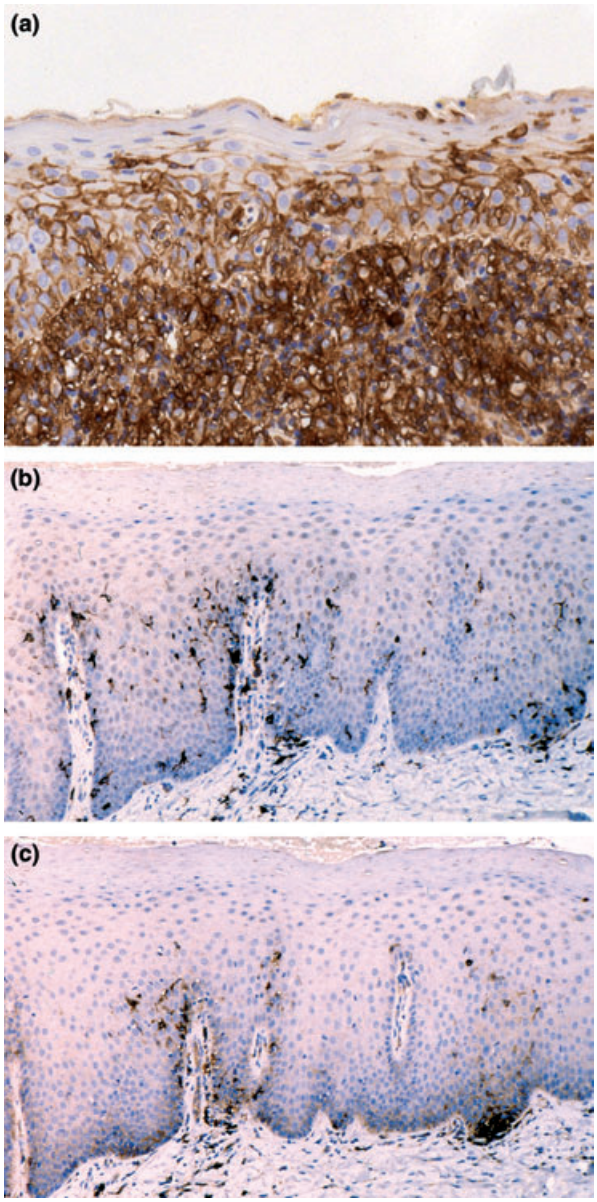

Figure 3 (a) Lichenoid reaction. Expression of CD40 in the stratum spinosum confers a reticular pattern. The inflammatory infiltrate is strongly positive. Field width: 450 μm. Serial sections of a non-specific hyperkeratosis stained for (b) CD1a and (c) CD40. CD1a-positive Langerhans cells outnumber those expressing CD40. Field width: 900 μm.

that of CD40-positive dendritic cells (Fig. 3c), with no obvious disease associations. Keratinocytes in the most superficial strata were CD40-negative. Lymphohistiocytic infiltrates in the lamina propria were strongly positive (Figs. 3a and c).

Epithelial dysplasia

Suprabasal membrane expression of CD40 was also more marked in most cases of epithelial dysplasia, though keratinocytes in the most superficial strata were again CD40-negative. However, there was absence of keratinocyte expression in nine of 33 (27.3%) epithelial dysplasias, one mild, one moderate and seven severe (Fig. 4).

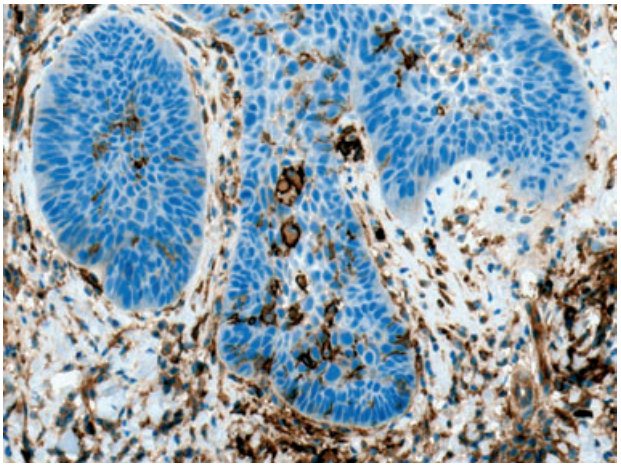

Figure 4 Severe epithelial dysplasia. CD40 expression by keratinocytes has been lost, with only the Langerhans cells positive within epithelium. Field width: 450 μm.

OSCC

CD40-staining was detected in 45 (69.2%) OSCC, comprising 17 of 21 (81.0%) of well differentiated, 22 of 32 (68.8%) of moderately differentiated and six of 12 (50.0%) of poorly differentiated tumours. However, there were different patterns of expression, none of which was consistently related to the degree of tumour differentiation. CD40 was expressed by all neoplastic keratinocytes in 29 (44.6%) OSCC (Fig. 5a). In nine (13.9%) tumours, all of which were well differentiated, CD40 was restricted to the peripheral strata of tumour islands, with loss of expression towards the keratinised centre (Fig. 5b). In seven (10.8%) OSCC, there was heterogeneous expression, with some islands positive, other islands showing zonal expression and some adopting the pattern seen in normal oral squamous epithelium (Fig. 5c). In the remaining 20 (30.8%) cases of OSCC, the tumour was CD40-negative, but there was variable expression by dendritic and stromal cells (Fig. 5d), and the overlying stratified squamous epithelium (Fig. 5e). Some positive OSCC arose from stratified squamous epithelium, which was negative (Fig. 5f).

Discussion

The present work is the most extensive survey of CD40 in oral mucosal epithelium. It demonstrates that CD40 is present in oral keratinocytes *in vitro*, and is expressed in the majority of oral stratified squamous epithelia, regardless of physiological or pathological status. This is consistent with previous studies (11–14, 22). While CD40-negative OSCC have not been previously reported, this is the first large series to be tested and in negative tumours, internal controls prove the staining to be genuine (Figs. 5c and d). Furthermore, SCC of the skin may be CD40-negative (8). The possible significance of absence of CD40 in oral keratinocytes, an observation that was made in approximately one-third of OSCC and a similar proportion of oral epithelial dysplasias (most of which were severe), is currently a matter of speculation. However, we have recently reported *in vitro* data on a CD40-negative oral keratinocyte line (15, 16). This line had a higher proliferation

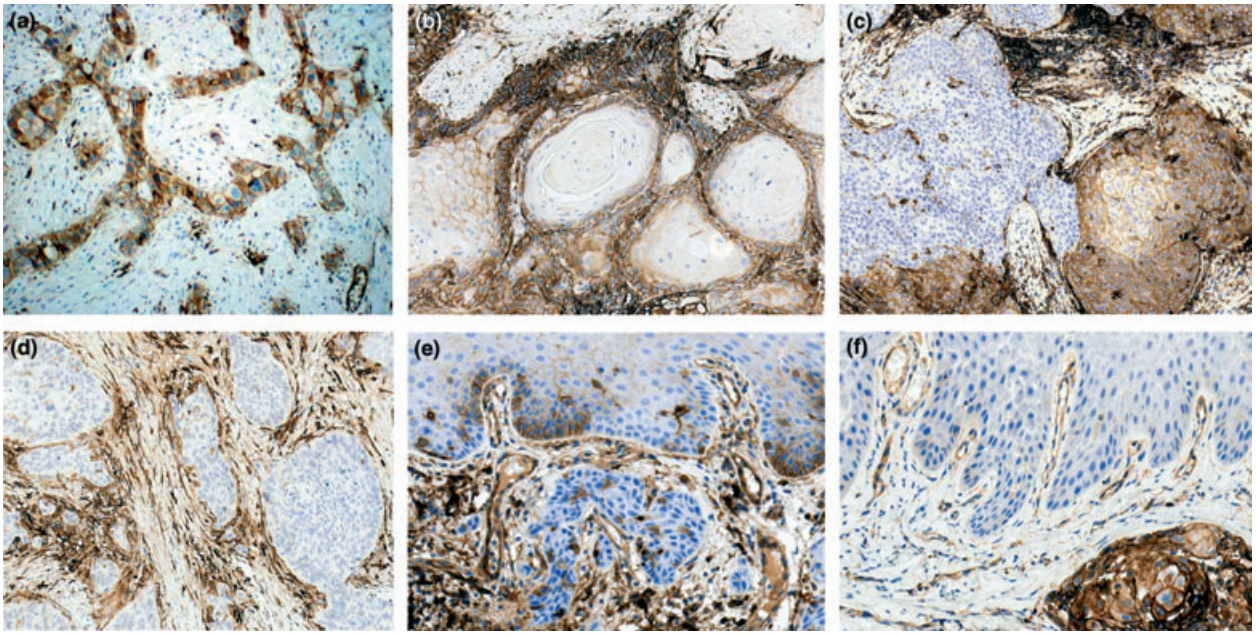

Figure 5 Expression of CD40 in OSCC. (a) a poor-moderately differentiated tumour, which is diffusely positive. Field width: 450 μm; (b) loss of expression of CD40 towards the centre of well-differentiated islands of tumour. The peripheral basal cells are positive. Field width: 900 μm; (c) biphasic expression in an OSCC showing negative, basaloid areas and positive, better-differentiated zones. Field width: 900 μm; (d) CD40-negative OSCC, surrounded by positive stroma. Field width: 900 μm; (e) invasive island of OSCC which, other than dendritic cells, is CD40-negative. In the overlying stratified squamous epithelium, there is focal absence of CD40 in the basal layer. Field width: 450 μm. (f) CD40-positive island of invasive OSCC deep to surface epithelium, which is negative. Field width: 450 μm.

rate than its CD40-transfected counterpart and was able to induce proliferation of allogeneic T lymphocytes, with concurrent upregulation of MHC class II and ICAM-1. However, unlike the CD40-transfected cells, CD40-negative keratinocytes did not show expression of CD86 and CD80, two other potent, membrane-bound co-stimulatory molecules.

CD40 expression in OSCC appears unrelated to tumour morphology. The CD40-negative neoplasm shown in Fig. 5d has the basaloid morphology typical of OSCC arising in the posterior oral cavity and oropharynx. However, other microscopically similar tumours were CD40-positive. It is nonetheless feasible that this morphological variant of OSCC is more prone to loss of CD40 than 'conventional' OSCC. As basal keratinocytes are CD40-positive in normal epidermis and oral epithelium (Fig. 2) (8–14), this is perhaps a paradox, but it is noteworthy that basal cell carcinomas of the skin may also be CD40-negative (8, 10). Whether the absence of CD40 in OSCC, or any of the staining patterns shown in Fig. 5, has any prognostic value is as yet unknown, as the tumours stained all developed in the last 3 years or less. Although follow-up data for the present cohort is limited, lymph node metastases have been found in the neck dissection specimens from patients with both CD40-positive and CD40-negative OSCC.

In conclusion, expression of CD40 by basal and parabasal oral keratinocytes is physiological, but it is lost in approximately one-third of oral epithelial dysplasias and OSCC. The significance of such loss remains unknown, but may be related to immunological or other abnormalities of keratinocyte homeostasis. The question remains as to whether this loss initiates pathological processes, or reflects them.

References

1. Vogel LA, Noelle RJ. CD40 and its crucial role as a member of the TNFR family. *Semin Immunol* 1998; **10**: 435–42.
2. Mazzei GJ, Edgerton MD, Losberger C, et al. Recombinant soluble trimeric CD40 ligand is biologically active. *J Biol Chem* 1995; **270**: 7025–8.
3. Tone M, Tone Y, Fairchild PJ, Wykes M, Waldmann H. Regulation of CD40 function by its isoforms generated through alternative splicing. *Proc Natl Acad Sci USA* 2001; **98**: 1751–6.
4. Grewal IS, Flavell RA. The role of CD40 ligand in costimulation and T-cell activation. *Immunol Rev* 1996; **153**: 85–106.
5. Grewal IS, Flavell RA. CD40 and CD154 in cell-mediated immunity. *Annu Rev Immunol* 1998; **16**: 111–35.
6. Dallman C, Johnson PW, Packham G. Differential regulation of cell survival by CD40. *Apoptosis* 2003; **8**: 45–53.
7. Afford SC, Ahmed-Choudhury J, Randhawa S, et al. CD40 activation-induced, Fas-dependent apoptosis and NF-kappaB/AP-1 signaling in human intrahepatic biliary epithelial cells. *FASEB J* 2001; **15**: 2345–54.
8. Viac J, Schmitt D, Claudy A. CD40 expression in epidermal tumors. *Anticancer Res* 1997; **17**: 569–72.
9. Peguet-Navarro J, Dalbiez-Gauthier C, Moulon C, et al. CD40 ligation of human keratinocytes inhibits their proliferation and induces their differentiation. *J Immunol* 1997; **158**: 144–52.
10. Kooy AJ, Prens EP, Van Heukelum A, Vuzevski VD, Van Joost T, Tank B. Interferon-gamma-induced ICAM-1 and CD40 expression, complete lack of HLA-DR and CD80 (B7.1), and inconsistent HLA-ABC expression in basal cell carcinoma: a possible role for interleukin-10? *J Pathol* 1999; **187**: 351–7.
11. Lang S, Whiteside TL, Lebeau A, Zeidler R, Mack B, Wollenberg B. Impairment of T-cell activation in head and neck cancer *in situ* and *in vitro*: strategies for an immune restoration. *Arch Otolaryngol Head Neck Surg* 1999; **125**: 82–8.
12. Posner MR, Cavacini LA, Upton MP, Tillman KC, Gornstein ER, Norris CM Jr. Surface membrane-expressed CD40 is present on tumor cells from squamous cell cancer of the head and neck *in vitro* and *in vivo* and regulates cell growth in tumor cell lines. *Clin Cancer Res* 1999; **5**: 2261–70.
13. Farmer I, Freysdottir J, Dalghous AM, Fortune F. Expression of adhesion and activation molecules in human buccal epithelial cell lines and normal human buccal epithelium *in situ*. *J Oral Pathol Med* 2001; **30**: 113–20.
14. Loro LL, Ohlsson M, Vintermyr OK, Liavaag PG, Jonsson R, Johannessen AC. Maintained CD40 and loss of polarised CD40 ligand expression in oral squamous cell carcinoma. *Anticancer Res* 2001; **21**: 113–7.
15. Villarroel Dorrego M, Whawell SA, Speight PM, Barrett AW. Transfection and ligation of CD40 in human oral keratinocytes affect proliferation, adhesion and migration but not apoptosis *in vitro*. *Clin Exp Dermatol* 2006; **31**: 266–71.
16. Villarroel Dorrego M, Whawell SA, Speight PM, Barrett AW. Transfection of CD40 in human oral keratinocytes upregulates immune potency and costimulatory molecules. *Brit J Dermatol* in press.
17. Savage NW, Seymour GJ, Kruger BJ. Expression of class I and class II major histocompatibility complex antigens on epithelial cells in recurrent aphthous stomatitis. *J Oral Pathol* 1986; **15**: 191–5.
18. Savage NW, Walsh LJ, Seymour GJ. Expression of class I and class II major histocompatibility complex antigens on oral mucosal epithelium. *J Oral Pathol* 1987; **16**: 153–7.
19. Farthing PM, Cruchley AT. Expression of MHC class II antigens (HLA DR, DP and DQ) by keratinocytes in oral lichen planus. *J Oral Pathol Med* 1989; **18**: 305–9.
20. Mutlu S, Matthews JB, Midda M, Scully C, Prime SS. MHC antigen expression in human oral squamous carcinoma cell lines. *J Pathol* 1991; **165**: 129–36.
21. Mutlu S, Scully C, Prime SS. Effect of IFN-gamma on the expression of MHC class I and class II antigens in a human malignant oral epithelial cell line. *J Oral Pathol Med* 1991; **20**: 218–21.
22. Villarroel Dorrego M, Speight PM, Barrett AW. Expression of Major Histocompatibility Complex class II and costimulatory molecules in oral carcinomas *in vitro*. *Med Oral Patol Oral Cir Bucal* 2005; **10**: 188–95.
23. Prime SS, Nixon SV, Crane IJ, et al. The behaviour of human oral squamous cell carcinoma in cell culture. *J Pathol* 1990; **160**: 259–69.
24. Thomas GJ, Lewis MP, Whawell SA, et al. Expression of the αvβ6 integrin promotes migration and invasion in squamous carcinoma cells. *J Invest Dermatol* 2001; **117**: 67–73.
25. Widney D, Boscardin WJ, Kasravi A, Martinez-Maza O. Expression and function of CD28 on Epstein-Barr virus-

positive B cell lines and AIDS-associated non-Hodgkin's lymphoma cell lines. *Tumour Biol* 2003; **24**: 82–93.
26. Miller WE, Cheshire JL, Raab-Traub N. Interaction of tumor necrosis factor receptor-associated factor signaling proteins with the latent membrane protein 1 PXQXT motif is essential for induction of epidermal growth factor receptor expression. *Mol Cell Biol* 1998; **18**: 2835–44.
27. Valle A, Zuber CE, Defrance T, Djossou O, De Rie M, Banchereau J. Activation of human B lymphocytes through CD40 and interleukin 4. *Eur J Immunol* 1989; **19**: 1463–7.
28. Krenacs L, Tiszalvicz L, Krenacs T, Boumsell L. Immunohistochemical detection of CD1a antigen in formalin-fixed and paraffin-embedded tissue sections with monoclonal antibody 010. *J Pathol* 1993; **171**: 99–104.
29. Vyberg M, Nielsen S. Dextran polymer conjugate two-step visualization system for immunohistochemistry. A comparison of EnVision+ with two three-step avidin–biotin techniques. *Applied Immunol* 1998; **6**: 3–10.

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