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ARTICLE *in* THE JOURNAL OF PATHOLOGY · JUNE 2010

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Journal of Pathology
J Pathol 2010; **221:** 153–163
Published online 3 February 2010 in Wiley InterScience
(www.interscience.wiley.com) DOI: 10.1002/path.2695

ORIGINAL PAPER

Functional expression of the chemokine receptor XCR1 on oral epithelial cells

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Abstract

Chemokines are chemoattractant cytokines which act on specific receptors and play an important role in leukocyte migration as well as physiological and pathological processes. We investigated the role of the chemokine receptor XCR1 and its ligand lymphotactin (Lptn/XCL1) in the regulation of oral epithelial cell behaviour. *In vitro* XCR1 mRNA and cell surface protein expression was detected in normal oral keratinocytes and oral squamous cell carcinoma cell lines. Lymphotactin mediated intracellular activation of the ERK1/2 signalling pathway and stimulated migration, invasion, and proliferation of all cells through XCR1. Oral cancer cells showed a greater response to lymphotactin than normal keratinocytes and a direct relationship between receptor expression and migration, invasion, and proliferation was observed. Exposure of normal keratinocytes to lymphotactin resulted in increased adhesion to fibronectin but not collagen and stimulated MMP-2 and MMP-9 but not MMP-7 release, whereas exposure of cancer cells resulted in increased adhesion to both collagen and fibronectin and stimulated production of MMP-2, MMP-9, and MMP-7. We observed XCR1 but not lymphotactin to be expressed by epithelial cells in normal oral mucosa *in vivo*, whilst both were expressed and up-regulated in inflammatory oral disease and oral cancer including primary and metastatic disease. Lymphotactin mRNA and constitutive intracellular protein were detected in normal keratinocytes and oral cancer cell lines *in vitro*. These findings show that XCR1 and its ligand, lymphotactin, are expressed by oral epithelial cells and suggest that they play a role in regulating the behaviour of these cells. Copyright © 2010 Pathological Society of Great Britain and Ireland. Published by John Wiley & Sons, Ltd.

Keywords: chemokine; chemokine receptor; oral cancer; XCR1; lymphotactin; oral keratinocytes

Received 16 July 2009; Revised 26 January 2010; Accepted 26 January 2010

No conflicts of interest were declared.

Introduction

Chemokines are a super-family of chemoattractant proteins first described in the immune system, where they mediate leukocyte migration and trafficking. They have been subdivided into four families on the basis of the relative position of their conserved two N-terminal cysteine residues [1]: CXCL (α), CCL (β), XCL (γ), and CX3CL (δ); and mediate their effects through interaction with specific G-protein-coupled receptors. It is now appreciated that chemokines have a critical role that extends beyond the regulation of lymphocyte migration and they influence a wide range of physiological and pathological processes. Expression of chemokine receptors and production of their ligands by epithelial cells have been demonstrated to be important in the maintenance of epithelial integrity by stimulating wound healing through their effects on cell proliferation and migration. In addition, expression on malignant epithelial cells has been shown to stimulate tumour invasion and progression and influence patterns of metastasis to different organs [2–9].

One chemokine which has not been reported on epithelial cells to date is XCR1. This receptor appears to be expressed only within the immune system and binds the chemokine lymphotactin (Lptn) [10]. Along with its receptor, Lptn is expressed within the lymphoid system and in addition to chemo-attraction of XCR1-expressing leukocytes, it influences T-cell proliferation [11] and regulation [12] as well as acting as an innate mucosal adjuvant by augmenting local and systemic antibody production [13]. In addition, it is up-regulated in a number of inflammatory conditions such as rheumatoid arthritis (RA) and Crohn's disease, as well as in tuberculosis [14–16]. Preliminary microarray data in our laboratory indicated that XCR1 may be present on both normal and malignant oral epithelial cells *in vitro*. Since intra-epithelial lymphocytes (IELs), which play an important role in epithelial homeostasis,

have been reported to express Lptn [15], this led us to investigate the possibility that oral epithelial behaviour may be regulated by Lptn. Here we show that XCR1 is expressed on oral epithelial cells *in vivo* in both health and disease, and that the receptor is functional and affects a number of epithelial functions.

Materials and methods

Immunohistochemistry for XCR1 and Lptn

Four-micrometre paraffin-embedded tissues sections were prepared from cases of normal oral mucosa ($n = 5$), oral lichen planus ($n = 5$), oral squamous cell carcinoma (OSCC; $n = 10$), and non-specific ulceration ($n = 5$) (South Sheffield Ethics Approval Committee Ref: 07/H1309/105). Mononuclear cells (MNCs) isolated from blood were stimulated with LPS (100 ng/ml; Peprotech, Rocky Hill, USA) for 2 h, fixed with 10% buffered formalin, clotted with fibrinogen and plasma, embedded in paraffin, and used as a positive control. Endogenous peroxidase was blocked using 2% hydrogen peroxide in methanol and sections were treated in citrate buffer (pH 6.0) in a microwave oven for 12 min for antigen retrieval before incubation with anti-human XCR1 antibody (Lifespan, Seattle, USA; 20 μg/ml) or anti-human Lptn antibody (Peprotech; 20 μg/ml) overnight at 4 °C. Rabbit serum served as a negative control for XCR1 and Lptn. In addition, Lptn antibody was pre-absorbed with recombinant Lptn (R&D Systems, Abingdon, UK) before application to determine specificity. Secondary antibody and avidin–biotin complex (ABC) provided with a Vectastain Elite ABC kit (Vector Labs, Burlingame, USA) were used in accordance with the manufacturer's instructions. A Vector NovaRed substrate kit (Vector Labs) was used to visualize peroxidase activity. Sections were then counterstained with haematoxylin, dehydrated, and mounted in DPX.

Cell culture

Normal oral keratinocytes (NOKs), human gingival fibroblasts (HGFs), and oral cancer cell lines, H357 (S. Prime, University of Bristol, UK), SCC4, TR146, CAL27, and FADU (ATCC, Teddington, UK), were used for this study. Normal keratinocytes and H357 cells were cultured in keratinocyte growth medium (KGM) [17]. Primary keratinocytes were grown from outgrowth cultures of mucosal biopsies independently of fibroblast feeder cells (Ethics Ref: STH 137 937). Primary HGFs were obtained from clinically healthy gingival tissue removed during third molar extractions. CAL27 and FADU were cultured in RPMI-1640 with 25 mM Hepes and L-glutamine (Invitrogen, Paisley, UK). DMEM (Invitrogen) was used for HGF and TR146, whereas SCC4 cells were grown in DMEM : F12 (1 : 1) with Hepes and L-glutamine (Invitrogen). Ten per cent fetal bovine serum (FBS), penicillin, and fungizone/amphotericin-B (Invitrogen) were added to all media unless specified otherwise.

RT-PCR for XCR1 and Lptn

Expression of XCR1 and its ligand Lptn at the mRNA level was studied using RT-PCR. Total RNA was isolated from cultured cells using Trizol (Ambion, Austin, TX, USA), DNase-treated to eliminate genomic DNA contamination, and reverse-transcribed using a Promega kit (Promega, Madison, USA). Neutrophil RNA (Ambion) was used as a positive control. The following primer sequences were used: XCR1: sense AGCTGGGGTCCCTACAACTT, anti-sense GACCCCCACGAAGACATAGA; Lptn: sense CCTCCTTGGCATCTGCTCTC, anti-sense ATTGGTCGATTGCTGGGTTC.

Each cycle consisted of denaturing at 94 °C (for 1 min), annealing at 60 °C (for 2 min), and extension at 72 °C (for 3 min) for a total of 35 cycles. A 1% agarose gel containing ethidium bromide was used to separate the PCR fragments and the gel was visualized and photographed using an imaging system (Syngene, Frederick, USA).

Immunocytochemistry for XCR1 and Lptn

Cells were grown on chamber slides (Becton Dickinson, San Jose, USA), washed with PBS, fixed in 2% paraformaldehyde, washed in PBS, and endogenous peroxidase was blocked with peroxidase-blocking solution (Dako, Glostrup, Denmark) for 10 min. Slides were incubated with rabbit anti-human XCR1 antibody (20 μg/ml) for 1 h and washed in PBS. Negative controls included omission of the primary antibody. Secondary antibody, ABC solution, and substrate solution (Vector Labs) were added as described for immunohistochemistry and the slides were counterstained with haematoxylin.

Intracellular expression of Lptn was assessed using immunofluorescence on cultured cell monolayers. Cells were grown on chamber slides (BD, Bedford, USA) and fixed/permeabilized with 2% paraformaldehyde containing 0.1% Triton-X100 (Sigma, Dorset, UK) for 10 min. Fixative was removed; slides were washed in PBS; and rabbit anti-human Lptn antibody (20 μg/ml) was added for 1 h. Negative controls received antibody pre-absorbed with recombinant Lptn. Excess antibody was removed and FITC-conjugated secondary antibody was applied (Dako; 1 : 20 dilution) for 30 min. Slides were washed again and mounted using Vectashield mounting medium containing DAPI (Vector Labs).

Flow cytometry

Single cell suspensions were prepared using a non-enzymatic cell dissociation buffer (Sigma) and re-suspended at a density of 1×10^6 cells in ice-cold wash buffer [PBS containing 0.1% bovine serum albumin (BSA)]. Cells were incubated with XCR1 antibody

(20 μg/ml) for 1 h at 4 °C, washed twice, and FITC-conjugated secondary antibody (Dako) was added at 4 °C in the dark. Data were acquired on a FACSCalibur (Becton Dickinson, Mountain View, USA) and analysed using FlowJo software (Tree Star, Ashland, USA). Negative controls received rabbit serum or irrelevant IgG (Dako); isolated neutrophils from blood served as the positive control.

Detection of ERK1/2 phosphorylation

A cell-based ELISA kit (Active Motif, Carlsbad, USA) was used to study intracellular ERK1/2 phosphorylation in response to Lptn in accordance with the manufacturer's instructions. The assay measures differences in absorbance in response to different treatments by utilizing specific antibodies against total and phosphorylated intracellular ERK. Cells were grown to 80% confluence on 96-well plates, serum-starved overnight and stimulated with Lptn (100 ng/ml) for 10 min, fixed with 2% paraformaldehyde, washed, and then permeabilized with 0.1% Triton-X100 (Sigma). Phorbol 12-myristat 13-acetate (PMA) (Sigma; 50 ng/ml) was used as a positive control; absence of Lptn as a negative control; and CCL25/TECK (100 ng/ml) (Peprotech) as an irrelevant chemokine.

The plate was washed and blocked (buffer provided with the kit), followed by the application of 40 μl of diluted primary antibody for phosphorylated and total ERK at 4 °C. The plate was sealed and incubated with the primary antibodies overnight at 4 °C. Unbound antibody was removed and 100 μl of a biotinylated secondary antibody was added for 1 h at room temperature with gentle agitation. After removal of the secondary antibody, cells were washed and then 100 μl of developing solution was added to each well. The plate was incubated for 10 min at room temperature, protected from direct light, and stop solution was added. Absorbance was read on a spectrophotometer at 450 nm.

Proliferation assay

Cells were grown on 96-well plates and exposed to Lptn (100 ng/ml) for 72 h. XCR1 antibody or an irrelevant antibody, CXCR1 (20 μg/ml) (R&D Systems, Abingdon, UK), was added to confirm the specificity of the assay. KGM served as a positive and TECK as an irrelevant control. After 72 h, cell-titre aqueous one proliferation reagent (Promega) was added to each well and the absorbance was recorded at 492 nm. All assays were performed in triplicate and a standard curve was constructed for each, enabling the cell number to be determined.

Migration assay

Chemotaxis assays were performed using Transwell® insert polycarbonate membranes (8 μm pore size; Corning, Amsterdam, The Netherlands) in 24-well plates. Inserts were coated with fibronectin (Sigma) and blocked with 0.5% BSA for 1 h. Cells were detached from monolayers, washed, and re-suspended in DMEM (Invitrogen) with 0.1% BSA with or without XCR1 antibody or an irrelevant antibody, CXCR1 (R&D Systems; 20 μg/ml), for 30 min. 1×10^5 cells were seeded in the top compartment and Lptn (100 ng/ml) was added to the lower chamber. An irrelevant chemokine (TECK) (100 ng/ml) was used in the control wells. After 4 h, non-migratory cells on the upper membrane surface were removed and migratory cells attached to the bottom surface of the membrane were fixed with 10% formalin and stained with 0.1% crystal violet for 2 min at room temperature. Migrated cells in five random fields were counted using a light microscope for each membrane and the mean number of cells per assay was calculated. All assays were performed in triplicate. Data were expressed as a migration index, defined as (number of cells migrating to fibronectin/number of cells migrating to test substance) $\times 100$.

Invasion assay

Invasion assays were performed using Bio-coat matrigel invasion chambers (Becton Dickinson) in 24-well plates. Serum-starved cells (1×10^5), with or without XCR1 antibody (20 μg/ml) or an irrelevant antibody (CXCR1; 20 μg/ml), were added to the upper compartment and Lptn (100 ng/ml) was added to the bottom. The irrelevant chemokine TECK (100 ng/ml) served as a negative control and 10% FBS as a positive control. The invasion chambers were incubated for 22 h and non-invasive cells (on the upper surface) were removed with a cotton swab. Invasive cells on the underside of the membrane were fixed, stained, and mounted as for the migration assay. Assays were performed in triplicate and invasive cells in five random fields were counted for each membrane and the mean number of cells per assay was calculated. Data were expressed as the mean number of invasive cells for each assay.

Adhesion assay

96-well tissue culture plates were coated with fibronectin or type I collagen (Sigma; 10 μg/ml) overnight at 4 °C. Negative control wells were not coated. Non-specific adhesion was blocked by incubating the plate for 1 h with PBS containing 1% BSA at 37 °C in a CO_2 incubator. Cells were non-enzymatically detached from monolayers and re-suspended in serum-free medium. Some cells were incubated with XCR1 antibody (20 μg/ml) or an irrelevant antibody for at least 30 min at 37 °C and then stimulated with Lptn (10, 100, and 500 ng/ml) or an irrelevant chemokine (TECK) (100 ng/ml) for 2 h at 37 °C with gentle agitation. 4×10^4 cells were seeded in each well and allowed to adhere for 1 h at 37 °C. Medium was aspirated and unbound cells were removed by washing twice with PBS. Fresh medium was added, followed by the addition of 20 μl of MTS (Promega) to each well. Absorbance was recorded at

492 nm and a standard curve for each assay was used to determine the number of cells. All assays were performed in triplicate.

Detection of matrix metalloproteinases

Recombinant Lptn and TNF-α (Peprotech) (100 ng/ml) were diluted in serum-free media and cells were stimulated for 72 h. The tissue culture supernatants were centrifuged to remove any cellular debris and secretion of total MMP-2, -7, and -9 was quantified by ELISA (R&D Systems) according to the manufacturer's instructions. The MMP concentration in the culture supernatant was estimated by interpolation from the calibration curve obtained from the MMP standard.

Statistical analysis

Data are presented as means ± SD. Paired Student's *t*-test was used to determine the significance of the obtained results for all quantitative assays.

Results

XCR1 mRNA and protein are present in NOKs and oral cancer cell lines *in vitro*

To confirm our preliminary micro-array data that XCR1 is expressed by epithelial cells *in vitro*, RT-PCR, flow cytometry, and immunocytochemistry were carried out on cultured cells. *XCR1* mRNA was observed in NOKs, HGFs, as well as the H357, SCC4, CAL27, FADU, and TR146 cancer cells (Figure 1A). Immunocytochemistry showed XCR1 protein expression on NOKs, HGFs, and H357, SCC4 (Figures 1C–1G), and TR146 cells (not shown). Cell surface and cytoplasmic staining was seen but numerous negative cells were consistently observed in NOK and H357 populations.

Flow cytometric analysis showed variable numbers and fluorescence intensity of XCR1-positive cells within the different populations (Figure 2A). Approximately 40% of NOKs, 60% of H357 cells, and more than 80% of SCC4, CAL27, and FADU cells were positive. The percentage was lower for the TR146 cells (18%) and HGFs (10%).

XCR1 is expressed *in vivo* in normal, neoplastic, and inflamed oral mucosa

Immunohistochemistry on tissue sections showed that XCR1 is also expressed by epithelial cells *in vivo* in a range of lesions including normal oral mucosa, oral cancer, lichen planus, and non-specific ulceration (Figures 1J–1N). In addition, fibroblasts, endothelial cells, and lymphocytes in the lamina propria also stained positively for XCR1.

In normal mucosa, XCR1 expression was observed in the cytoplasm of basal keratinocytes with weak surface staining suprabasally (Figure 1J). In oral cancer, there appeared to be an increase in staining intensity on the cell surface and within the cytoplasm, both in primary and in metastatic tumours (Figures 1K and 1L). This increase was also seen in the basal keratinocytes in oral lichen planus and non-specific ulceration (Figures 1M and 1N).

Exposure to Lptn significantly up-regulates intracellular ERK1/2 phosphorylation and stimulates proliferation, migration, and invasion

Exposure of cells to Lptn resulted in a significant up-regulation of intracellular ERK1/2 phosphorylation in a dose-dependent manner ($p < 0.05$) (Figure 2B). Phosphorylated ERK1/2 levels in H357 and SCC4 cells were significantly higher than those in NOKs at all doses ($p < 0.05$). Stimulation of SCC4 cells with Lptn also resulted in intracellular calcium mobilization (data not shown).

Lptn caused a significant and specific increase in the proliferation of NOKs, H357, and SCC4 cells compared with unstimulated cells ($p < 0.05$). The proliferative effect of Lptn was significantly reduced by an antibody against XCR1 but not by the irrelevant CXCR1 antibody ($p < 0.05$) (Figure 2C). Migration of NOKs, H357, and SCC4 cells was significantly enhanced in the presence of Lptn, compared with fibronectin alone ($p < 0.05$) (Figure 3A). This Lptn-mediated migration was significantly and specifically reduced by prior incubation with XCR1 antibody ($p < 0.05$). The percentage increase in migration of the different cell lines correlated with the proportion of positive cells as determined by flow cytometry. Lptn also stimulated invasion of NOKs, H357, and SCC4 cells ($p < 0.05$), which was almost completely blocked by XCR1 but not CXCR1 antibody (Figure 3B). The percentage increase in invasion correlated with the proportion of XCR1-positive cells determined by flow cytometry.

Lptn increases adhesion of NOKs to fibronectin and oral cancer cells to fibronectin and collagen through XCR1

Epithelial cell adhesion to extracellular matrix (ECM) components is a fundamental aspect of cell migration and invasion, and interaction of chemokines with their receptors has been shown to influence this in wound healing as well as in cancer [2,18]. Fibronectin is an important component of the ECM which binds collagen and cell surface integrins, mediating reorganization of the cytoskeleton and facilitating cell movement.

Stimulation of NOKs, H357, and SCC4 cells with Lptn resulted in a significant increase in adhesion to fibronectin in a dose-dependent manner ($p < 0.05$), which was completely inhibited by pre-incubation with XCR1-blocking antibody ($p < 0.05$) (Figure 4A). Lptn facilitated significantly greater adhesion of H357 and SCC4 cells to fibronectin than NOKs at all doses ($p < 0.05$). No difference was observed between the adhesion of H357 and SCC4 cells.

Adhesion of both H357 and SCC4 cells to collagen increased significantly in a dose-dependent manner

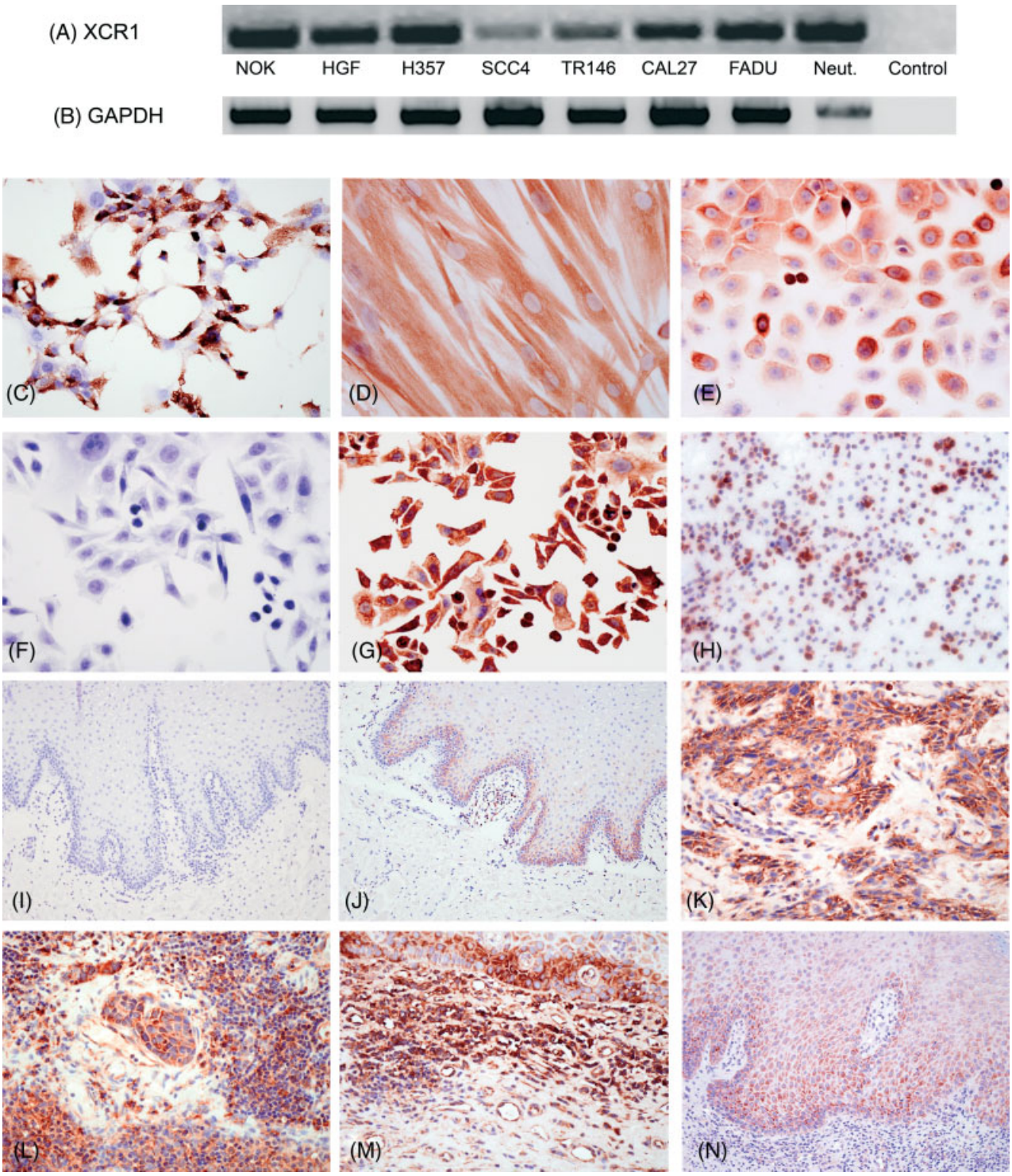


Figure 1. (A) mRNA expression of XCR1 *in vitro* (representative picture). Band indicates a PCR product of 169 bp; the control lane received no RNA. (B) GAPDH loading controls. (C–G) Representative pictures showing *in vitro* immunocytochemical XCR1 expression. Original magnification ×60. No staining was observed in the negative controls (F). Staining was seen on the cell surface and in the cytoplasm in NOKs (C), HGFs (D), H357 (E), and SCC4 cells (G). Negative cells were also observed in NOKs (C) and H357 cells (E). SCC4 negative control (F). (H–N) *In vivo* expression of XCR1 in isolated mononuclear cells (H), normal oral mucosa (J), oral squamous cell carcinoma (K, L), lichen planus (M), and non-specific ulceration (N) as shown by immunohistochemistry (representative sections; original magnification ×40). No staining was observed in the negative controls (I). Staining for XCR1 was seen in the keratinocytes in the epithelium (J, M, N), some of the infiltrating lymphocytes (J, M, N), invading tumour cells (K), and metastatic deposits in regional lymph nodes (L). Staining was performed at least three times for every sample.

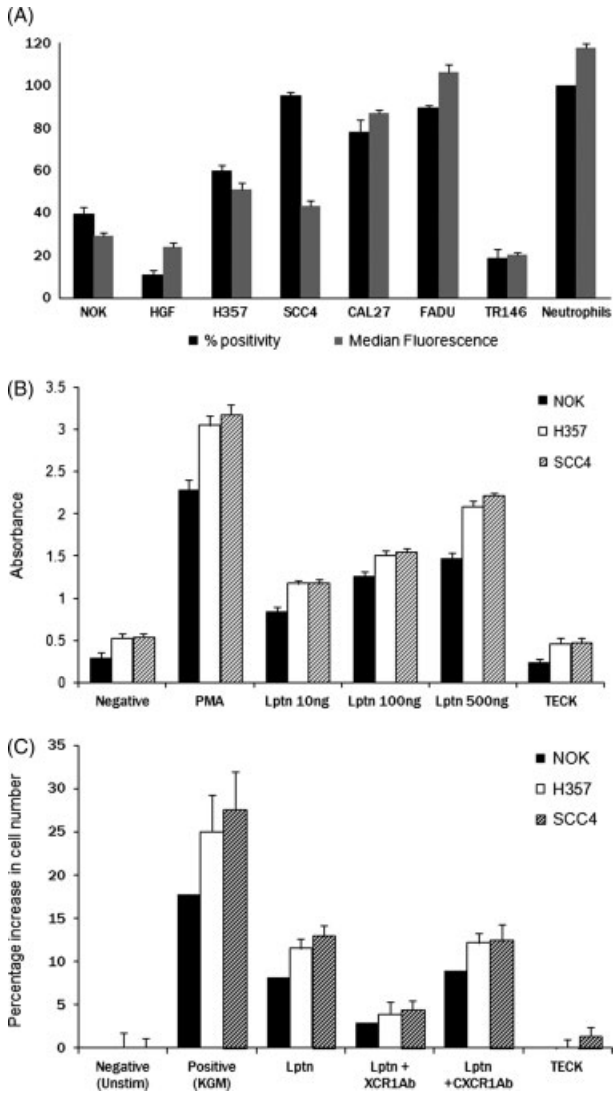


Figure 2. (A) Percentage of XCR1-positive cells and median fluorescence intensity as determined by flow cytometry (mean of three independent experiments ± SD). (B) A cell-based ELISA showing comparison of changes in absorbance related to Lptn-mediated intracellular ERK1/2 phosphorylation in NOKs, H357, and SCC4 cells using specific antibodies ($OD_{450\ nm}$ ± SD). (C) An MTS-based assay showing comparison of XCR1 Lptn-mediated proliferation between NOKs, H357, and SCC4 cells (mean ± SD) as determined indirectly using a standard curve. n = mean of three independent experiments; TECK = irrelevant control chemokine; negative = additive-free medium.

after exposure to Lptn (Figure 4B), and this was significantly and specifically inhibited by pre-incubation with XCR1 antibody. No difference was detected between the adhesion of H357 and SCC4 cells, and no increase in the adhesion of NOKs to collagen was observed after incubation with Lptn.

MMP-2 and MMP-9 are constitutively produced by NOKs and oral cancer cells; Lptn significantly up-regulates MMP-2 and MMP-9 release through XCR1

The migration and invasion of epithelial cells are also facilitated by the production of matrix metalloproteinases (MMPs), which are a family of enzymes that modify the ECM [19–21]. The role of chemokines in MMP regulation is well documented in a range of cancers [22–24]. Constitutive MMP-2 and MMP-9 production was seen in NOKs, H357 and SCC4 cells but the production of these MMPs by H357 and SCC4 cells was significantly higher than that by NOKs ($p < 0.05$) (Figures 5A and 5B). A significant increase in MMP-2 and MMP-9 release was seen in all cells after Lptn stimulation ($p < 0.05$) but this was significantly higher in H357 and SCC4 cells compared with NOKs ($p < 0.05$). Addition of XCR1 antibody significantly reduced MMP-2 and MMP-9 expression from all Lptn-stimulated cells compared with the irrelevant CXCR1 antibody ($p < 0.05$).

Exposure to Lptn triggers XRC1-dependent MMP-7 release from oral cancer cells but not from NOKs

No constitutive or Lptn-stimulated expression of MMP-7 by NOKs was seen. In contrast, addition of TNF-α or Lptn caused a significant increase in MMP-7 production from H357 and SCC4 cells (Figure 5C), although the levels were significantly higher for the SCC4 than for the H357 cells ($p < 0.01$). This increase in MMP-7 production was specifically inhibited by XCR1 antibody.

Lptn expression *in vitro* and *in vivo*

The results reported so far show that oral epithelial cells respond to Lptn but its source *in vivo* in the oral cavity is not known. Immunohistochemical staining showed no evidence of Lptn production in normal oral mucosa (Figure 6B). However, in oral cancer, Lptn staining was seen in the epithelium of primary and metastatic tumours (Figures 6D and 6E), as well as in the adjacent epithelium (Figure 6C) and by lymphocytes in the regional nodes (Figure 6F). Lptn expression was not restricted to oral cancer and was also seen in reactive inflammatory lesions including lichen planus and non-specific ulceration (Figures 6G and 6H).

To determine whether Lptn is produced by epithelial cells *in vitro* as well as *in vivo*, RT-PCR and protein analysis were performed. *Lptn* mRNA was seen in NOKs, HGFs, and all cancer cell lines tested (Figure 6M). Immunofluorescence staining demonstrated constitutive intracellular localization of Lptn in NOKs, H357, and SCC4 cells (Figures 6I–6L). These results were corroborated by flow cytometry (data not shown).

Discussion

The data presented in this study show that XCR1 and its ligand Lptn are expressed by normal and malignant oral epithelial cells, and demonstrate their expression outside the immune system for the first time. Furthermore, we have shown that epithelial XCR1 is

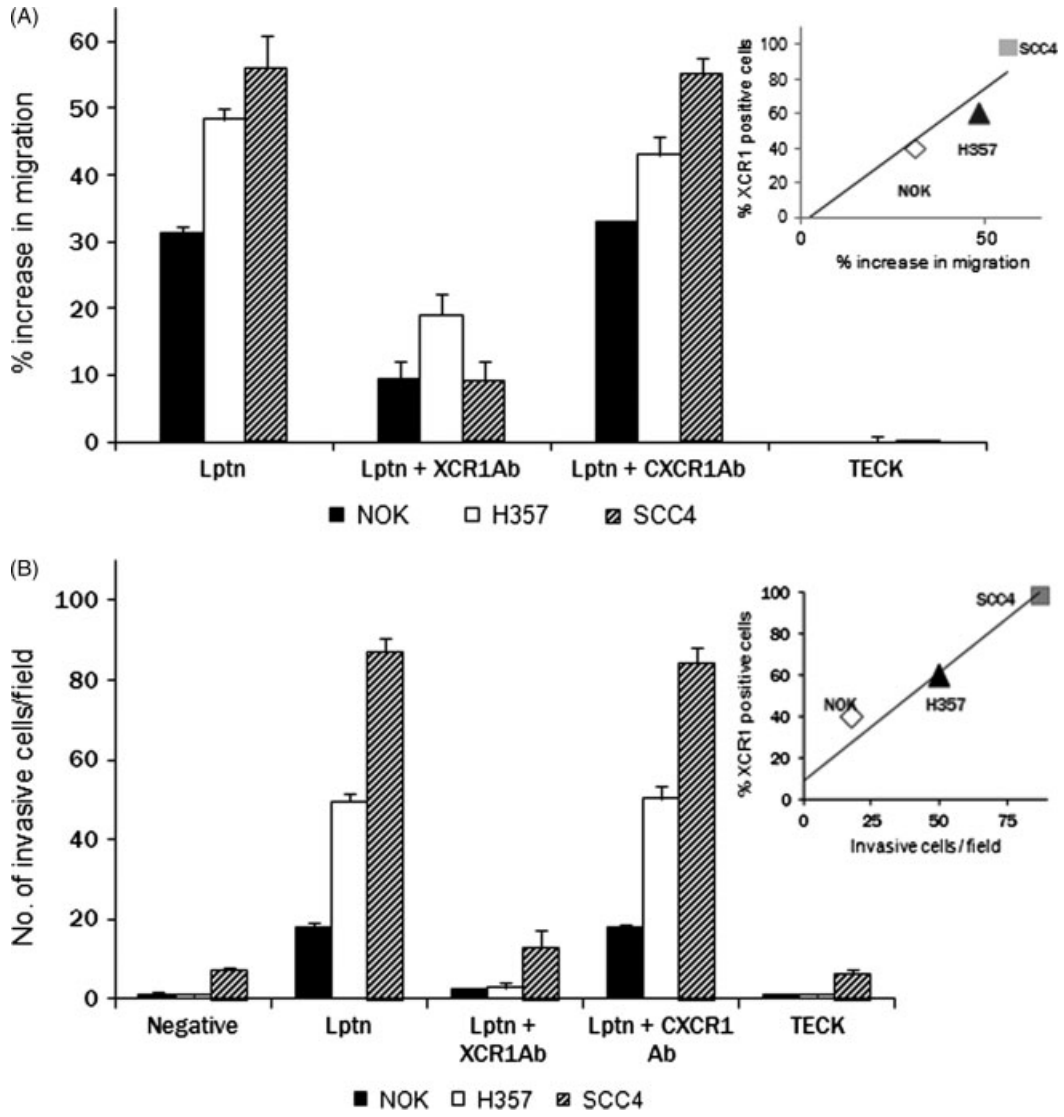


Figure 3. Comparison of the percentage increase in migration (A) and invasion (B) of NOKs, H357, and SCC4 cells towards Lptn (n = mean of three independent experiments done in triplicate, ± SD). The inset graphs show the relationship between % XCR1-positive cells and the number of cells that migrated or invaded towards Lptn.

functional, since Lptn significantly increased cell proliferation in addition to stimulating migration, invasion, adhesion to ECM proteins, and MMP production. These effects were significantly and specifically inhibited by an antibody against XCR1.

XCR1 expression was observed at both the mRNA and the protein level in NOKs and cancer cell lines. In general, a higher percentage of cancer cells than NOKs were positive for XCR1 but exposure to Lptn facilitated migration, invasion, and proliferation in both. However, cancer cells showed a greater quantitative response than NOKs in all experiments, indicating a correlation with the number of XCR1-positive cells and providing further evidence that the effects of Lptn are mediated through XCR1. Lptn also increased intracellular ERK1/2 phosphorylation in both NOKs and cancer cell lines, indicating that signal transduction has taken place through RAS-GTP and MEK1/2. However, it is not clear which downstream transcription factors are important in mediating the effects of Lptn on proliferation, migration, and invasion.

Lptn also mediated an increase in adhesion of both NOKs and oral cancer cells to fibronectin but only increased adhesion of the cancer cells to collagen. The mechanism underlying this increase is not clear but it is possible that Lptn may affect the expression or avidity of integrins involved in binding to fibronectin (eg $\alpha5\beta1$, $\alpha v\beta6$) and type I collagen ($\alpha1\beta1$, $\alpha2\beta1$), and there may be differences in cell surface integrin expression between NOKs and cancer cells. These results suggest that normal and cancer cells respond to Lptn in a different manner and that XCR1 may mediate a stronger adhesive, chemotactic, and invasive response in cancer than normal cells. The chemokine receptor CXCR4 has been shown to mediate tumour cell adhesion to ECM components (fibronectin, collagen, and laminin) in prostate cancer by up-regulation of integrins $\alpha5$ and $\beta3$ [25]. In addition, binding of tumour cells to the ECM can up-regulate the expression of

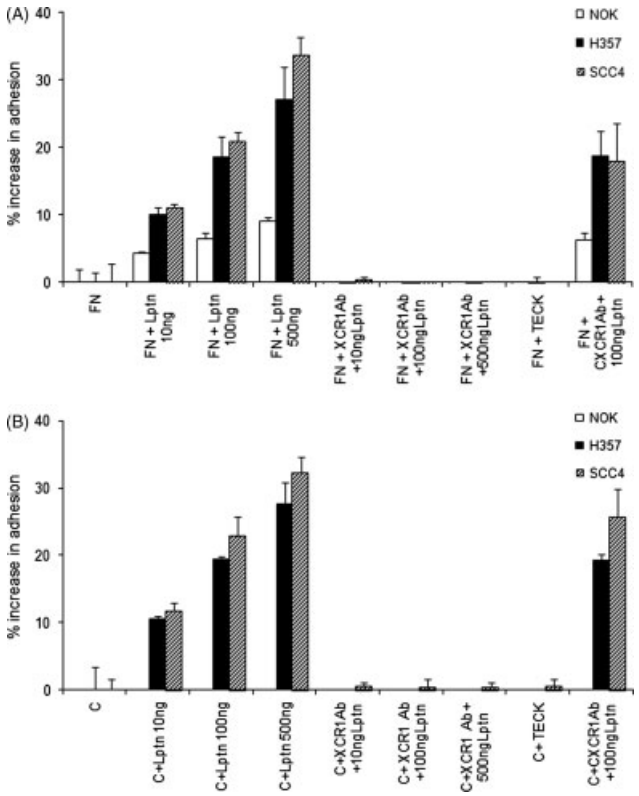


Figure 4. Comparison of the percentage increase in adhesion of NOKs, H357, and SCC4 cells to fibronectin (A) and collagen (B) after Lptn stimulation (n = mean of three independent experiments done in triplicate, ± SD). C = collagen; Lptn concentration is in ng/ml; TECK = irrelevant chemokine.

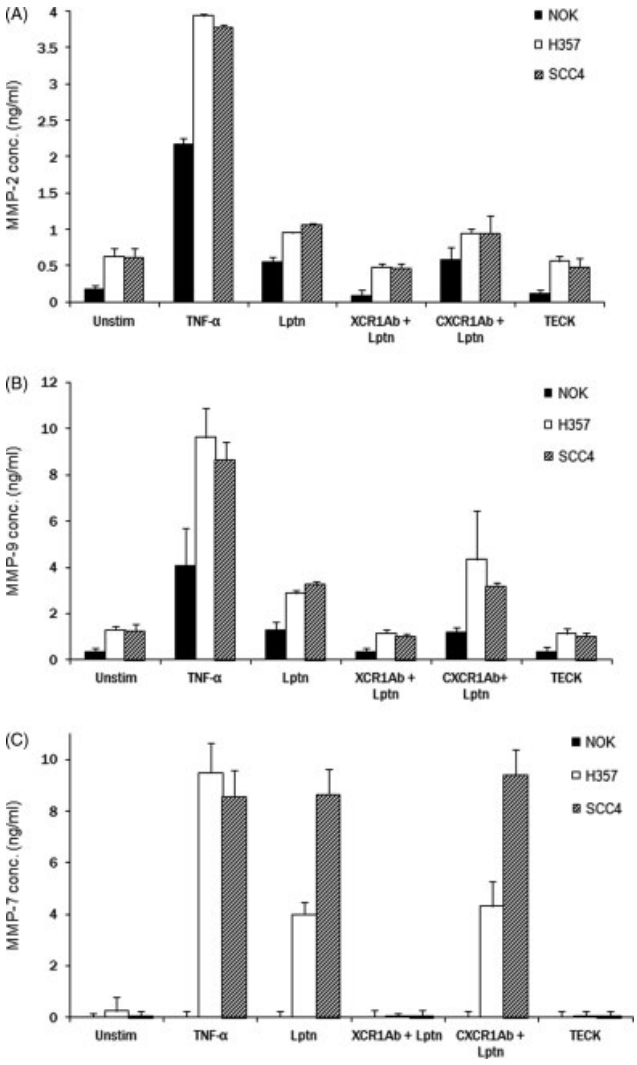


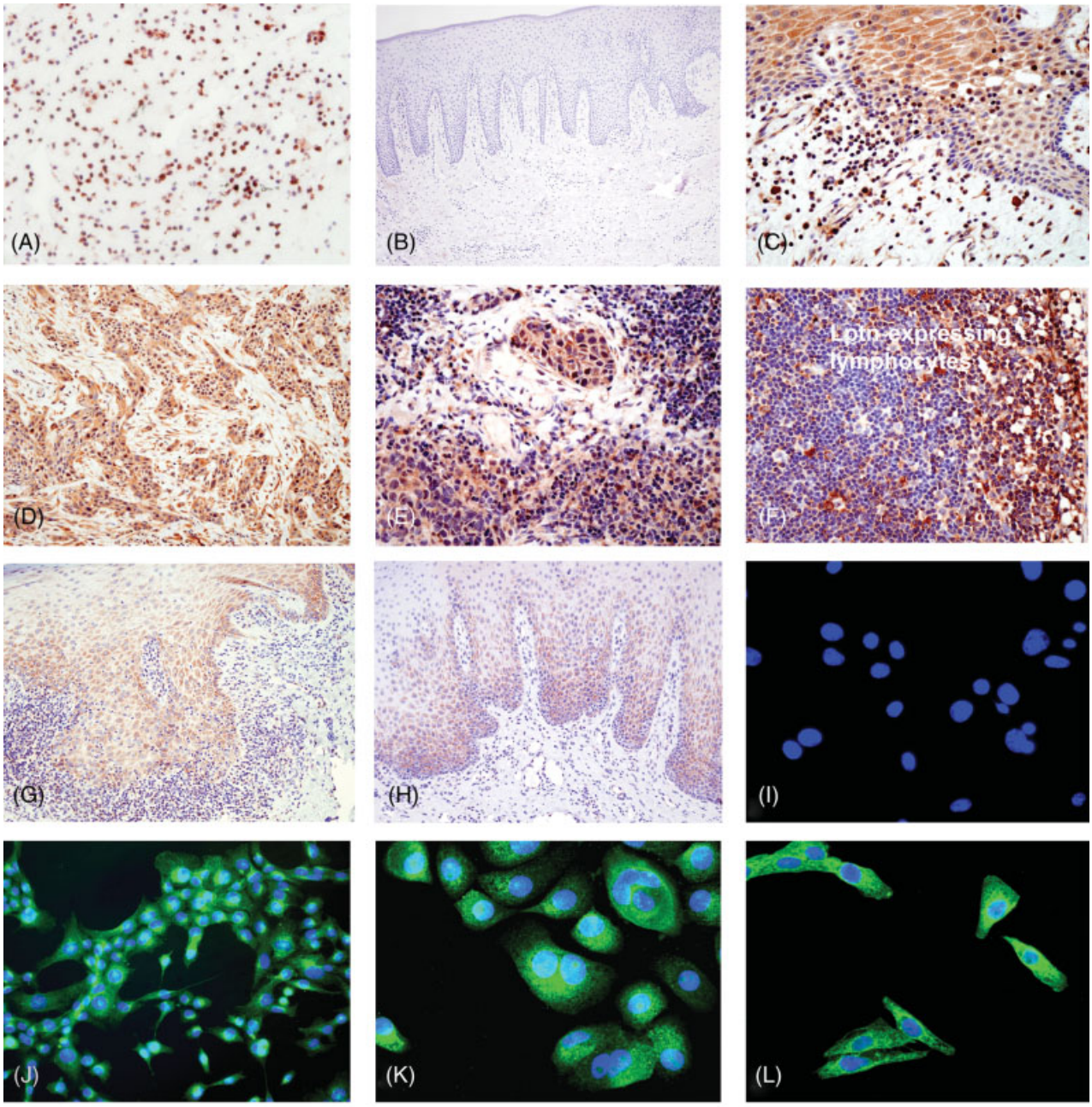
Figure 5. Comparison of MMP-2 (A), MMP-9 (B), and MMP-7 (C) production between NOKs, H357, and SCC4 cells (n = mean of three different experiments done in triplicate, ± SD). TECK = irrelevant chemokine.

chemokine receptors and their ligands, thus providing an autocrine mechanism of activation. For example, growth of pancreatic tumour cells on laminin-1 up-regulates CXCR4 (mediated by the α6β1 integrin) and CXCL8 (IL-8) expression (mediated by α6β1 and α3β1 integrins) [26]. It is not known whether XCR1 is up-regulated on oral cancer cells upon binding to ECM proteins.

A difference in MMP production between normal and cancer cells was also noticed. XCR1/Lptn facilitated a small increase in MMP-2 and MMP-9 from NOKs and this was more pronounced in cancer cells. In contrast to our findings, Lptn has been previously shown to down-regulate MMP-2 production from synoviocytes in culture [14]. This difference may be explained, at least in part, by the difference in origin of the cells between the two studies. Expression of MMP-2 and MMP-9 by oral epithelial cells has been reported previously, which is in agreement with our findings [27,28]. Moreover, chemokines have been shown to influence MMP production in other cancers. For instance, CXCL12 (SDF-1α) up-regulates MMP-9 expression in colorectal cancer [29] and myeloma [30], leading to an increase in the migration and invasion of these cells. In addition, Yuecheng *et al* showed that CXCL12 also facilitates increased invasion of ovarian cancer cells through up-regulation of MMP-2 and MMP-9 [31].

Interestingly, Lptn increased the secretion of MMP-7 in cancer cells but not NOKs and this was markedly higher in SCC4 than in H357 cells. This increase in MMP-7 production from SCC4 cells correlates with XCR1 positivity and suggests that XCR1/Lptn interaction may be more important in the production of MMP-7 than in that of MMP-2 or MMP-9. A role for MMP-7 in the biology of oral cancer has previously been reported [32,33], and expression of MMP-7 is significantly correlated with lymph node metastasis and patient survival [34]. Our findings raise the possibility that XCR1/Lptn may play a role in this process by stimulating MMP-7 production.

Further evidence for a possible role for XCR1/Lptn in oral cancer metastasis comes from our *in vivo* findings. XCR1 expression appeared much stronger in oral cancer than in normal oral epithelium and was present not only in primary, but also in metastatic tumours. Furthermore, many lymphocytes within the nodes were Lptn-positive. Thus, it is possible that, as has been postulated for other chemokines, Lptn



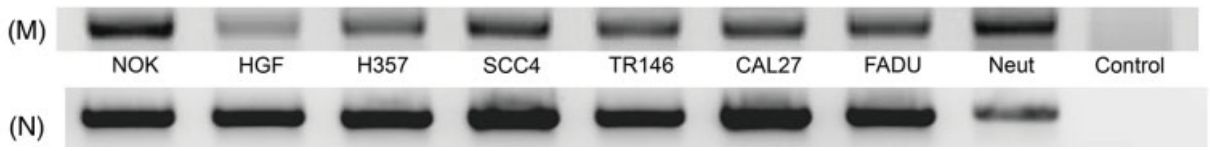


Figure 6. (A–H) Immunohistochemical expression of Lptn in isolated mononuclear cells/control (A), normal oral mucosa (B), epithelium adjacent to oral squamous cell carcinoma (C), oral squamous cell carcinoma (D, E), regional lymph nodes (F), lichen planus (G), and non-specific ulceration (H). Representative sections; original magnification ×40. No staining was observed in the negative controls (not shown) or normal oral mucosa. Staining for Lptn was seen in the epithelium (C), IELs (C), infiltrating lymphocytes (C, G), invading tumour cells (D), and metastatic deposits in regional lymph nodes (E). Staining was performed at least three times for every sample. (I–L) Representative pictures showing *in vitro* intracellular expression of Lptn in NOKs, H357, and SCC4 cells as shown by immunofluorescence. Original magnification ×60. No staining was observed in the negative control (I). Intracellular staining for Lptn was seen in NOKs (J), H357 (K), and SCC4 (L) cells. (M) mRNA expression of Lptn (representative picture); the control lane received no RNA. Band indicates a PCR product of 301 bp. (N) GAPDH loading controls.

production within regional lymph nodes may provide a chemoattractant gradient to XCR1-positive tumour cells in the lymphatics. However, our results are based on a limited number of cases and further work is required to determine whether XCR1 expression is correlated with lymph node metastasis. The situation is also complicated by the apparent production of Lptn by epithelial cells and lymphocytes at the primary site. It is possible that locally produced Lptn stimulates both tumour growth, through its effects on proliferation, and spread, through its effects on migration, invasion, and MMP production. However, Lptn has been shown to facilitate chemotaxis of cytotoxic T lymphocytes and these could exert an anti-tumour influence [35–38]. These opposing potential effects, combined with the possibility that the stimulated lymphocytic infiltrate releases other cytokines or growth factors which may play a role in Lptn or epithelial cell regulation, indicate the complexity of the scenario. Further work is required to understand the precise role and regulation of XCR1 and Lptn in oral squamous cell carcinoma.

Epithelial cells in normal oral mucosa *in vivo* expressed XCR1 but did not stain for Lptn and this raises a question about the function of XCR1 and the source of Lptn in healthy mucosa. Previously, IELs within murine epidermis have been shown to produce Lptn *in vivo* [39]. However, we did not observe Lptn expression on IELs in normal oral mucosa. The reason for this difference is not clear and further work is required to determine whether this is a species difference, although it should be noted that Lptn has been detected in the IELs in human colon [15].

Interestingly, both epithelial XCR1 and Lptn appeared to be up-regulated *in vivo* in the inflammatory conditions of lichen planus and non-specific ulceration, indicating that up-regulation is not restricted to OSCC. In addition, both XCR1 and Lptn appeared to be expressed by endothelial cells and fibroblasts. At present, we only have confirmatory *in vitro* data for *XCR1* and *Lptn* mRNA expression by human gingival fibroblasts, and further work is required to assess expression by endothelial cells. It is possible that XCR1 and Lptn play a role in wound healing by increasing proliferation and migration across the ulcer surface, helped in part by the increase in MMP production and adhesion to fibronectin which was observed in this study. Another possible function of Lptn may be the attraction of neutrophils and other inflammatory cells once the ulceration has occurred. In oral lichen planus, destruction of the basal cells is associated with a dense lymphocyte-dominated infiltrate in the lamina propria. Lptn may play a role in mediating the attraction of this infiltrate but may also promote healing by stimulating the proliferation and migration of epithelial cells.

In summary, our results have shown the expression of functional XCR1 and its ligand Lptn by normal and malignant oral epithelial cells for the first time. This suggests that expression is not restricted to the immune system, a finding that is supported by a recent study showing that XCR1 is expressed by melanocytic lesions [40]. Details of the significance of this expression in terms of normal and malignant epithelial cell behaviour remain to be determined.

Acknowledgment

This study was supported by the University of Sheffield; the Higher Education Commission, Pakistan; and the British Society for Oral and Maxillofacial Pathology.

References

1. Baggiolini M, Dewald B, Moser B. Human chemokines: an update. *Annu Rev Immunol* 1997; **15**: 675–705.
2. Raja, Sivamani K, Garcia MS, Isseroff RR. Wound re-epithelialization: modulating kerationcyte migration in wound healing. *Front Biosci* 2007; **12**: 2849–2868.
3. Burger JA, Kipps TJ. CXCR4: a key receptor in the crosstalk between tumor cells and their microenvironment. *Blood* 2006; **107**: 1761–1767.
4. Wang X, Wang E, Kavanagh JJ, Freedman RS. Ovarian cancer, the coagulation pathway, and inflammation. *J Transl Med* 2005; **3**: 25.
5. Anders HJ, Vielhauer V, Schlondorff D. Chemokines and chemokine receptors are involved in the resolution or progression of renal disease. *Kidney Int* 2003; **63**: 401–415.
6. Wilson J, Balkwill F. The role of cytokines in the epithelial cancer microenvironment. *Semin Cancer Biol* 2002; **12**: 113–120.
7. Gillitzer R, Goebeler M. Chemokines in cutaneous wound healing. *J Leukoc Biol* 2001; **69**: 513–521.
8. Mahalingam S, Karupiah G. Chemokines and chemokine receptors in infectious diseases. *Immunol Cell Biol* 1999; **77**: 469–475.
9. MacDermott RP. Chemokines in the inflammatory bowel diseases. *J Clin Immunol* 1999; **19**: 266–272.
10. Yoshida T, Imai T, Kakizaki M, Nishimura M, Takagi S, Yoshie O. Identification of single C motif-1/lymphotactin receptor XCR1. *J Biol Chem* 1998; **273**: 16551–16554.
11. Cerdan C, Serfling E, Olive D. The C-class chemokine, lymphotactin, impairs the induction of Th1-type lymphokines in human CD4(+) T cells. *Blood* 2000; **96**: 420–428.
12. Cerdan C, Devilard E, Xerri L, Olive D. The C-class chemokine lymphotactin costimulates the apoptosis of human CD4(+) T cells. *Blood* 2001; **97**: 2205–2212.
13. Lillard JW Jr, Boyaka PN, Hedrick JA, Zlotnik A, McGhee JR. Lymphotactin acts as an innate mucosal adjuvant. *J Immunol* 1999; **162**: 1959–1965.
14. Blaschke S, Middel P, Dorner BG, Blaschke V, Hummel KM, Kroczek RA, *et al*. Expression of activation-induced, T cell-derived, and chemokine-related cytokine/lymphotactin and its functional role in rheumatoid arthritis. *Arthritis Rheum* 2003; **48**: 1858–1872.
15. Middel P, Thelen P, Blaschke S, Polzien F, Reich K, Blaschke V, *et al*. Expression of the T-cell chemoattractant chemokine lymphotactin in Crohn's disease. *Am J Pathol* 2001; **159**: 1751–1761.
16. Rosas-Taraco AG, Higgins DM, Sanchez-Campillo J, Lee EJ, Orme IM, Gonzalez-Juarrero M. Intrapulmonary delivery of XCL1-targeting siRNA in mice chronically infected with *Mycobacterium* tuberculosis. *Am J Respir Cell Mol Biol* 2009; **41**: 136–145.
17. Formanek M, Millesi W, Willheim M, Scheiner O, Kornfehl J. Optimized growth medium for primary culture of human oral keratinocytes. *Int J Oral Maxillofac Surg* 1996; **25**: 157–160.
18. Shulby SA, Dolloff NG, Stearns ME, Meucci O, Fatatis A. CX3CR1-fractalkine expression regulates cellular mechanisms

involved in adhesion, migration, and survival of human prostate cancer cells. *Cancer Res* 2004; **64**: 4693–4698.
19. Stamenkovic I. Extracellular matrix remodelling: the role of matrix metalloproteinases. *J Pathol* 2003; **200**: 448–464.
20. Folgueras AR, Pendas AM, Sanchez LM, Lopez-Otin C. Matrix metalloproteinases in cancer: from new functions to improved inhibition strategies. *Int J Dev Biol* 2004; **48**: 411–424.
21. Bjorklund M, Koivunen E. Gelatinase-mediated migration and invasion of cancer cells. *Biochim Biophys Acta* 2005; **1755**: 37–69.
22. Wu X, Fan J, Wang X, Zhou J, Qiu S, Yu Y, *et al*. Downregulation of CCR1 inhibits human hepatocellular carcinoma cell invasion. *Biochem Biophys Res Commun* 2007; **355**: 866–871.
23. Redondo-Munoz J, Terol MJ, Garcia-Marco JA, Garcia-Pardo A. Matrix metalloproteinase-9 is up-regulated by CCL21/CCR7 interaction via extracellular signal-regulated kinase-1/2 signaling and is involved in CCL21-driven B-cell chronic lymphocytic leukemia cell invasion and migration. *Blood* 2008; **111**: 383–386.
24. Yuecheng Y, Xiaoyan X. Stromal-cell derived factor-1 regulates epithelial ovarian cancer cell invasion by activating matrix metalloproteinase-9 and matrix metalloproteinase-2. *Eur J Cancer Prev* 2007; **16**: 430–435.
25. Engl T, Relja B, Marian D, Blumenberg C, Muller I, Beecken WD, *et al*. CXCR4 chemokine receptor mediates prostate tumor cell adhesion through alpha5 and beta3 integrins. *Neoplasia* 2006; **8**: 290–301.
26. Grzesiak JJ, Smith KC, Burton DW, Deftos LJ, Bouvet M. Integrin-mediated laminin-1 adhesion upregulates CXCR4 and IL-8 expression in pancreatic cancer cells. *Surgery* 2007; **141**: 804–814.
27. Bennett JH, Morgan MJ, Whawell SA, Atkin P, Roblin P, Furness J, *et al*. Metalloproteinase expression in normal and malignant oral keratinocytes: stimulation of MMP-2 and -9 by scatter factor. *Eur J Oral Sci* 2000; **108**: 281–291.
28. Robinson CM, Stone AM, Shields JD, Huntley S, Paterson IC, Prime SS. Functional significance of MMP-2 and MMP-9 expression by human malignant oral keratinocyte cell lines. *Arch Oral Biol* 2003; **48**: 779–786.
29. Brand S, Dambacher J, Beigel F, Olszak T, Diebold J, Otte JM, *et al*. CXCR4 and CXCL12 are inversely expressed in colorectal cancer cells and modulate cancer cell migration, invasion and MMP-9 activation. *Exp Cell Res* 2005; **310**: 117–130.
30. Parmo-Cabanas M, Molina-Ortiz I, Matias-Roman S, Garcia-Bernal D, Carvajal-Vergara X, Valle I, *et al*. Role of metalloproteinases MMP-9 and MT1-MMP in CXCL12-promoted myeloma cell invasion across basement membranes. *J Pathol* 2006; **208**: 108–118.
31. Yuecheng Y, Xiaoyan X. Stromal-cell derived factor-1 regulates epithelial ovarian cancer cell invasion by activating matrix metalloproteinase-9 and matrix metalloproteinase-2. *Eur J Cancer Prev* 2007; **16**: 430–435.
32. Birkedal-Hansen B, Pavelic ZP, Gluckman JL, Stambrook P, Li YQ, Stetler-Stevenson WG. MMP and TIMP gene expression in head and neck squamous cell carcinomas and adjacent tissues. *Oral Dis* 2000; **6**: 376–382.
33. Impola U, Uitto VJ, Hietanen J, Hakkinen L, Zhang L, Larjava H, *et al*. Differential expression of matrilysin-1 (MMP-7), 92 kD gelatinase (MMP-9), and metalloelastase (MMP-12) in oral verrucous and squamous cell cancer. *J Pathol* 2004; **202**: 14–22.
34. de Vicente JC, Lequerica-Fernandez P, Santamaria J, Fresno MF. Expression of MMP-7 and MT1-MMP in oral squamous cell carcinoma as predictive indicator for tumor invasion and prognosis. *J Oral Pathol Med* 2007; **36**: 415–424.
35. Cairns CM, Gordon JR, Li F, Baca-Estrada ME, Moyana T, Xiang J. Lymphotactin expression by engineered myeloma cells drives tumor regression: mediation by CD4+ and CD8+ T cells and neutrophils expressing XCR1 receptor. *J Immunol* 2001; **167**: 57–65.
36. Cao X, Zhang W, He L, Xie Z, Ma S, Tao Q, *et al*. Lymphotactin gene-modified bone marrow dendritic cells act as more potent adjuvants for peptide delivery to induce specific antitumor immunity. *J Immunol* 1998; **161**: 6238–6244.
37. Sheng XL, Zhang H. *In-vitro* activation of cytotoxic T lymphocytes by fusion of mouse hepatocellular carcinoma cells and lymphotactin gene-modified dendritic cells. *World J Gastroenterol* 2007; **13**: 5944–5950.
38. Wang Q, Yu H, Zhang L, Ju D, Pan J, Xia D, *et al*. Adenovirus-mediated intratumoral lymphotactin gene transfer potentiates the antibody-targeted superantigen therapy of cancer. *J Mol Med* 2002; **80**: 585–594.
39. Boismenu R, Feng L, Xia YY, Chang JC, Havran WL. Chemokine expression by intraepithelial gamma delta T cells. Implications for the recruitment of inflammatory cells to damaged epithelia. *J Immunol* 1996; **157**: 985–992.
40. Seidl H, Richtig E, Tilz H, Stefan M, Schmidbauer U, Asslaber M, *et al*. Profiles of chemokine receptors in melanocytic lesions: *de novo* expression of CXCR6 in melanoma. *Hum Pathol* 2007; **38**: 768–780.