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Ligation of CD24 expressed by oral epithelial cells induces kinase dependent decrease in paracellular permeability mediated by tight junction proteins

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ABSTRACT

In previous studies we demonstrated uniform strong expression of CD24 in the epithelial attachment to the tooth and in the migrating epithelium of the periodontitis lesion. Titers of serum antibodies auto-reactive with CD24 peptide correlated with reduced severity of periodontal disease. In the present study an epithelial culture model with close correspondence for expression patterns for tight junction components in periodontal epithelia was used. Ligation of CD24 expressed by oral epithelial cells with an anti-CD24 antibody induced formation of tight junctions and live-cell imaging confirmed that paracellular diffusion of fluorochrome-labeled dextran was reduced. Expression of mRNA and protein for zona occludens-1, -2, junction adhesion molecule-A (JAM-A), occludin and claudins-1, -4, -8, -15, -18 was significantly increased following ligation of CD24 but only claudins-4 and -15, JAM-A, occludin and zona occludens-1 and -2 were increased at cell contacts. This change in expression patterns reflected that observed between the epithelium of the periodontal lesion and that of the healthy gingival attachment. In the model system, response profiles to kinase inhibitors indicated a key role of c-Src kinase activation in the development of diffusion-limiting tight junction complexes. Activation was confirmed by demonstrating concomitant phosphorylation of the kinase. Pre-incubation with antibodies against JAM-A and claudin-15 prevented barrier-enhancing effects of anti-CD24 antibodies while pre-incubation with antibody to claudin-4 was partially effective. It is concluded that antibodies to CD24 facilitate expression and location of JAM-A, claudins-4 and -15 that mediate enhanced epithelial barrier function in a protective response against bacterial products.

1. Introduction

In highly polarized epithelia such as the intestinal mucosa, tight junction complexes, organized as a continuous band, the zona occludens, restrict permeability to low molecular weight products [1]. Components of the intercellular tight junction structure include occludin [2], junction adhesion molecules (JAM)-A, -B and -C [3] and the claudins [4]. The claudin composition of the tight junction defines the particular properties of the junction [5], with claudins-1, -2, -3, -4 and -7 reported to be prominent in stratified epithelia [6]. Important scaffolding cytoplasmic proteins, especially ZO-1, -2 and -3, bind to claudins and occludin strands [7].

Tight junction formation in normal stratified epithelia, metaplastic stratified epithelia and cultured derivatives of these tissues, has been a subject of controversy but is supported by recent studies [8–10]. Functional studies have shown that claudin-1 expression regulates the permeability barrier of the epidermis of newborn mice [11]. Langbein et al. [10] have described a range of morphological types of close intercellular contacts associated with localization of tight junction proteins in diverse mucosal sites including oral mucosa. Contrasting with the zona occludens organization of highly polarized epithelia, less polarized stratified epithelia typically display a tight junction arrangement of point contacts or maculae occludens [12]. It is possible that as yet unrecognized additional functions of tight junction proteins both regulate epithelial biology and modulate barrier function within stratified epithelium [12].

CD24 is a heavily glycosylated protein ligand for vascular P-selectin and is anchored by phosphoinositol linkage to lipid rafts within the cell membrane [13]. It has been shown to be a regulator of the chemokine receptor CXCR4 [14]. Recent analysis has indicated that CD24 critically mediates a protective effect against tissue injury [15]. Experimentally, barrier function of model epithelial monolayers was enhanced by challenge with monoclonal antibody against CD24 [16]. This report examines the mechanism of the enhanced barrier function mediated by CD24.

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2. Materials and methods

2.1. Oral epithelial culture

The epithelial cell line (H413) derived from a human oral squamous cell carcinoma [17], displays stratified epithelial cell morphology in culture. H413 clonal lines were established using a limiting dilution method in our laboratory as described previously [18]. Barrier function of tight junctions in oral epithelial cells was measured by plating H413 clone-1 cells in 24 mm Transwell filters on 0.4 μm polyester membranes (Corning Incorporated Life Science, USA) as described previously [16]. Briefly, H413 clone-1 cells were cultured in a low Ca^{2+} medium and passaged onto 24 mm Transwell filters. Triplicate confluent monolayers ($2 \times 10^5/cm^2$) were exposed to 5 μg/ml antibody to CD24 peptide, or isotype IgG1 negative control antibody, or isotype IgG1 negative control antibody plus Src-kinase inhibitors; herbimycin A (100 nM, Sigma), genistein (10 μM, Sigma), PP3 (100 nM, 1 μM, Calbiochem, Melbourne, Australia), PP2 (100 nM, 1 μM, Calbiochem), Src kinase inhibitor saracatinib (AZD0530, 1 μM, provided by Astra Zeneca Ltd.), respectively, or CD24 peptide antibody plus each inhibitor as above. Dextran Alexa Fluor 647 (10 kDa wt. Molecular Probes, Invitrogen) diluted 1:50 from a stock solution of 1 mg/ml in medium was added to each well. At various time points after commencing the experiments, 50 μl media were taken from each lower and higher compartment, and analyzed for fluorescence using a Perkin–Elmer LS50B luminescence spectrometer, Ex650 nm/Em668 nm for Alexa Fluor 647. Diffusion of labeled dextran was determined as moles of fluorophore transferred to the lower compartment calculated by reference to a standard curve. Data from three independent experiments were analyzed by paired *t*-test.

2.2. Analysis for c-Src kinase by Western blot

H413 clone-1 cells grown to confluence in low Ca^{2+} medium in 75 cm^2 flasks (Sarstedt Australia Pty Ltd., South Australia) were challenged either with isotype control antibody or anti-CD24 peptide antibody (5 μg/ml) for 3 h. Cultures were washed in PBS, harvested by scraper, and extracted in 0.5% Triton-X100 in PBS pH 7.4 with a proteinase inhibitor cocktail (Sigma cat. P8340). Protein concentrations were adjusted to be equal, and 2-fold diluted proteins were resolved on a 12% PAGE gel stained with Coomassie blue to confirm comparable sample loading. Equivalent preparations were resolved on PAGE gel, transferred to nitrocellulose membranes (Bio-Rad) and blocked with 3% bovine serum albumin (Sigma) in TBS pH 7.4 overnight. Membranes were probed with either rabbit polyclonal anti-Src antibody (Abcam, Cambridge UK ab7950) diluted 1:500 from stock of 200 μg/ml or rabbit polyclonal anti-Src phospho Y418 antibody (Abcam, Cambridge UK ab4816) diluted 1:1000 from stock solution (100 μg/ml), both provided by the manufacturer. Membranes were washed and incubated with the second antibody, AP-conjugated goat anti-rabbit IgG (DAKO). Blots were developed in AP substrate (Bio-Rad).

2.3. Affinity isolation of c-Src kinase

Cell lysates (in 0.5% Triton-X100) were clarified by centrifugation and the supernatants added to the affinity matrix. NHS-Sepharose beads (GE Life Sciences) were activated according to the manufacturer's specifications for ligation of anti-c-Src kinase antibody, using 80 μg of antibody per 0.5 ml of packed beads. The beads were washed extensively in Tris buffer to block unreacted sites; aliquots (0.25 ml) were then incubated with rotation with 0.3 ml of cell lysate (representing the harvest from one flask for each of test and control adjusted to achieve equal protein concentrations) overnight at 4 °C. Beads were washed extensively in PBS containing 1 M NaCl and extracted directly by boiling in SDS sample buffer with 2-mercaptoethanol and resolved on 12% PAGE gel in preparation for Western blotting as described above.

2.4. RNA extraction and reverse transcription

Comprehensive analysis of expression of genes encoding tight junction components was performed. Briefly, sub-confluent H413 clone-1 cells (25 cm^2 flask containing 5×10^6 cells) were incubated with one of the following for 3 h:5 μg/ml of CD24 mouse monoclonal (ALB9) peptide antibody (IgG1, Abcam Ltd., Cambridge, UK); which recognizes a short non-glycosylated peptide sequence close to the site of GPI linkage of the protein core of the cluster-w4/CD24 antigen [19]; treated with CD24 antibody plus a representative Src-protein kinase inhibitor saracatinib (AZD0530, 1 μM); with an IgG1 negative control (DAKO, Denmark); or with IgG1 negative control antibody plus saracatinib (AZD0530, 1 μM). Cells were harvested by scraper in PBS and pelleted by centrifugation. Trizol (1 ml) was added to the cell pellet (5×10^6) for homogenization and extraction in chloroform and isopropanol. RNA pellets were washed in 75% (v/v) ethanol, centrifuged, air dried and resuspended in an appropriate volume of DEPC-treated MilliQ water. For reverse transcription, the First-Strand cDNAs were synthesized with oligo$(dT)_{12-18}$ (Invitrogen), 10 mM dNTP (Promega), RNaseOUT™ Recombinant RNase Inhibitor (Invitrogen) and SuperScript™ III Reverse Transcriptase (Invitrogen) according to the manufacturer's (Invitrogen) protocol.

2.5. Quantitative real-time RT-PCR analysis of expression of genes encoding tight junction components

Primers for genes encoding claudins, occludin, JAMs and ZO-1, -2, -3 (see Supplementary Table 1) were designed using Oligo Explorer software (1.1.0) and synthesised by Sigma. Real-time RT-PCR analyses were performed by SYBR Green based assays using the Stratagene MxPro-Mx3005P System and software (MxPro 4.10). PCR reaction was conducted with 2 μl of diluted cDNA samples, 200 nM of each respective forward and reverse primer in a 25 μl final reaction mixture with Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen). cDNA samples isolated from non-manipulated H413 clone-1 cells were quantified by PicoGreen kit (Invitrogen) and then used for constructing standard curves (2000–2 pg) by reference to the expression of the house keeping gene encoding β-actin. The PCR reaction for each gene was carried out in triplicate in 96-well plates, and initiated by activation at 95 °C for 2 min, followed by 40 PCR cycles of denaturation at 95 °C for 15 s, annealing and extension at 60 °C for 30 s. Altered gene expression was analyzed by paired *t*-tests. A level of $P < 0.05$ was accepted as statistically significant.

2.6. The impact of tight junction proteins claudins-4, -15 and JAM-A on barrier function induced by anti-CD24 antibody

JAM-A, claudins-15 and -4 were analyzed. Triplicate confluent monolayers ($2 \times 10^5/cm^2$) in transfer wells were stimulated using 5 μg/ml antibody to CD24 peptide, or isotype IgG1 negative control, or CD24 peptide antibody plus polyclonal antibody to JAM-A or claudins-4 or -15, and simultaneously together with low molecular weight dextran Alexa Fluor 647 (10 kDa wt. Molecular Probes, Invitrogen) diluted 1:50 from a stock solution of 1 mg/ml in medium. At various time points (1–7, 9, 12 h) after commencing the experiments, 50 μl media were taken from each lower and higher compartment, and analyzed for fluorescence using a Perkin–Elmer