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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

LS50B luminescence spectrometer, Ex650 nm/Em668 nm for Alexa Fluor 647. Transfer of labeled dextran was calculated as moles of fluorophore transferred to the lower compartment determined by reference to a standard curve. Data from three independent experiments were analyzed by paired *t*-test.

2.7. Statistical analysis

All data were analyzed by paired *t*-test (mean ± S.D., two-tailed, 95% CI range) from at least three consecutive experiments for real-time RT-PCR, Western blots, and permeability assays where necessary. A level of $P < 0.05$ was accepted as statistically significant.

3. Results

3.1. Inhibitors of Src kinases ablate the effect of anti-CD24 peptide antibody on barrier function of epithelial monolayers

The broad spectrum kinase inhibitor genistein was effective in suppressing the barrier promoting effect of anti-CD24 antibody whereas PP3, which does not inhibit Src kinases, had no detectable effect. Mediation by c-Src kinase was suggested by near complete inhibition of the permeability reducing effect of anti-CD24 peptide antibody by addition of herbimycin A at 100 nM (Supplementary Fig. 1A). CD24 receptor expression in the presence of herbimycin A was monitored over time to exclude the possibility that down-regulation of CD24 was a component of the effect of the inhibitor (Supplementary Fig. 1B). Further indication of the essential role for c-Src kinase was obtained by demonstrating that saracatinib blocked anti-CD24 enhancement of barrier function (Fig. 1). Live cell imaging indicated these effects were related to paracellular diffusion of fluorochrome-dextran (Supplementary Fig. 1C). To confirm activation of c-Src kinase in response to anti-CD24, cell lysates were probed for c-Src kinase and Src phospho tyr418 in Western blots. The results shown in Fig. 2 indicate that relative to isotype antibody control treatment, there was an increase in c-Src kinase in cultures challenged with anti-CD24 peptide antibody (Fig. 2A). Strong staining for affinity-isolated c-Src phospho tyr418 (Fig. 2B) indicated the kinase had been activated after allowance for protein concentrations determined by sample loading controls (Fig. 2C) and β-actin loading controls (Fig. 2D). The combined data implicate activation of c-Src kinase as a mediator of the effect of ligand binding to CD24 on the assembly and function of effective barrier function in this epithelial model.

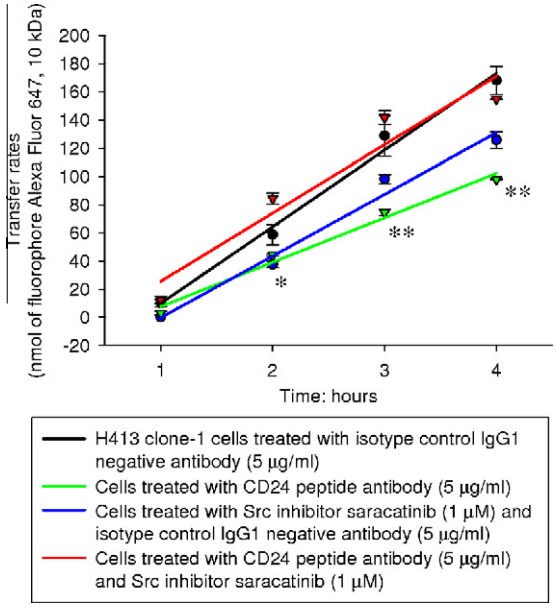

Fig. 1. Effect of the c-Src kinase inhibitor saracatinib (AZD0530) on permeability of CD24 peptide antibody treated H413 epithelial monolayers. A regression analysis representative of three independent experiments for time points from triplicate cultures (showing mean values ± S.D.) is shown. Cultures were permeable for the translocation of low molecular weight dextran (Alexa Fluor 647 10 kDa, * refers to $^{*}P < 0.05$, $^{**}P < 0.01$, paired *t*-test).

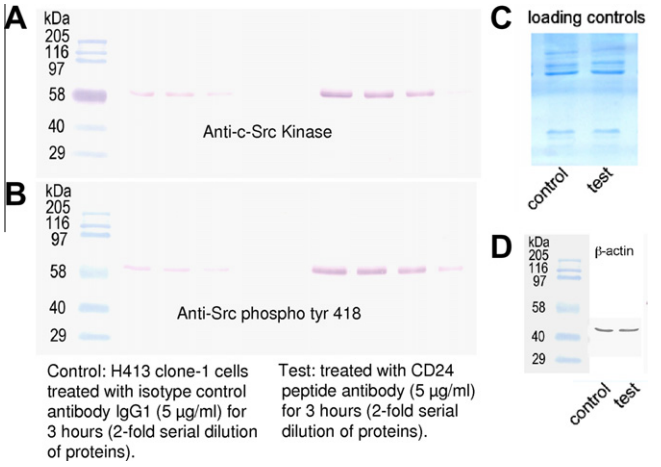

Fig. 2. Western blot analysis of Src kinase in H413 clone-1 cells. Cell lysates were adjusted for protein concentration and matching SDS–PAGE loading control. (A) Anti-c-Src kinase showing increased amount of the kinase in cultures treated with anti-CD24 peptide antibody compared to cultures treated with isotype control antibody. (B) Anti-Src phospho tyr418 antibody showing strong reaction characteristic of active kinase in cultures treated with anti-CD24 peptide antibody. To confirm specificity for c-Src kinase, this enzyme was affinity isolated from cell lysates as described in methods. Western blot of the recovered antigen showed identical staining patterns to those displayed in (A) and (B) (data not shown). (C) SDS–PAGE sample loading controls stained with Coomassie blue from identical gels which were transferred onto nitrocellulose membrane. (D) β-Actin loading control.

3.2. Up-regulation of expression of genes encoding multiple tight junction components by anti-CD24 peptide antibody

Quantitative real-time RT-PCR findings shown in Fig. 3 indicate that incubation with anti-CD24 antibody induced significant up-regulation of genes encoding most claudins, occludin, ZO-1, -2, -3 and JAM-A, -B, -C; there was no increase in expression levels of genes encoding claudins-7 and -11. No expression was detected for genes encoding claudins-3, -5, -10b (isoform-2), -18A1.1 (isoform-1), -19 and -23 in this model.

Active concentrations of saracatinib (AZD0530) had no impact on enhanced gene expression induced by anti-CD24 antibody (data not shown).

To study corresponding patterns of protein expression, semi-quantitative analysis of tight junction proteins was performed by densitometry scans of Western blots developed under standardized conditions. Cell lysates were probed with antibodies to tight junction proteins in Western blot analysis to confirm the increase in gene expression was manifest as up-regulation of tight junction proteins. Data indicate a significant increase in levels of claudins-1, -4, occludin, claudins-8, -18 JAM-A, and claudin-15 in cultures challenged with anti-CD24 peptide antibody. However, levels of claudins-2 and -10 were reduced by exposure to anti-CD24 antibody. After challenge with both anti-CD24 peptide antibody and the c-Src kinase inhibitor saracatinib levels of claudin-1, occludin, and claudin-15 were down-regulated while claudin-2 was up-regulated (Supplementary Fig. 2).

Confocal microscopy was employed to localize tight junction protein expression in cultures challenged with anti-CD24 peptide antibody or with both anti-CD24 peptide antibody and saracatinib.

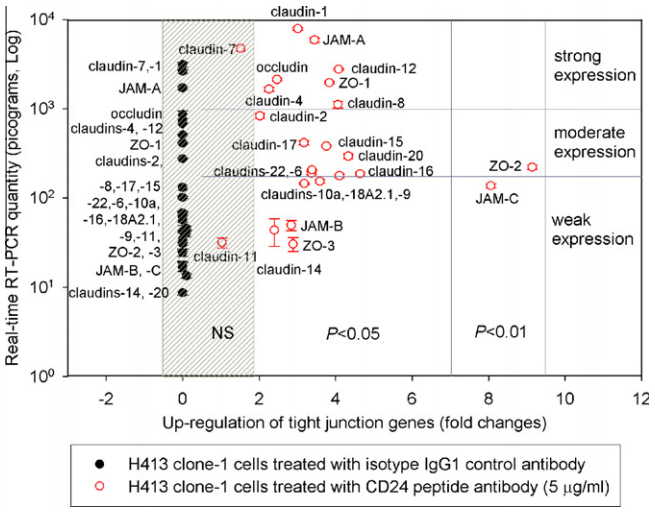

Fig. 3. Regulation of expression of genes encoding tight junction proteins by anti-CD24 peptide antibody. Quantitative real-time RT-PCR findings indicated significant up-regulation ($P < 0.05$) following a 3 h incubation period and strong expression (>10^3 pg) of genes encoding claudins-1, -4, -8, -12, occludin, ZO-1 and JAM-A; strong expression (>10^3 pg) but no increase in claudin-7. Moderate expression (10^2–10^3 pg) and up-regulation of genes encoding claudins-2, -6, -15, -16, -17, -20, -22 ($P < 0.05$), and ZO-2 ($P < 0.01$). Weak expression (10–10^2 pg) and up-regulation of genes encoding claudins-9, -10a (isoform-1), -14, -18A2.1 (isoform-2), ZO-3, JAM-B ($P < 0.05$), and JAM-C ($P < 0.01$), weak expression (10–10^2 pg) but no increase in claudin-11. No expression was detected for claudins-3, -5, -10b (isoform-2), -18A1.1 (isoform-1), -19 and -23 in this cell model.

Claudins-4, -15, and JAM-A localized at cell borders (Supplementary Fig. 3). A summary of data from Western blot and confocal microscopy is shown in Supplementary Table 2.

3.3. Antibodies to claudins-4, -15 and JAM-A reduce the barrier effect of anti-CD24 peptide antibody

Collectively, the data indicated that claudins-4, -15 and JAM-A were strongly implicated in mediation of the barrier promoting effect of anti-CD24 antibody. To evaluate the contribution of these components permeability assays were performed in the presence of specific antibodies to these products. The data indicate that antibodies to JAM-A and claudin-15 completely inhibited the effect of anti-CD24 peptide antibody on barrier function. Antibody to claudin-4 partially inhibited the anti-CD24 effect (Fig. 4).

4. Discussion

Ligation of CD24 expressed by H413 oral epithelial cells induced both increased expression and peripheral localization of selected proteins associated with tight junctions. This altered expression profile for tight junction components correlated with reduced paracellular transfer of low molecular weight dextran across H413 cell monolayers.

The role of c-Src kinase activation in mediating the CD24 signal in cultured oral epithelial cells was confirmed by demonstrating loss of barrier function following incubation with the selective c-Src kinase inhibitor saracatinib [20] and increased levels of c-Src phospho-tyrosine 418 [21] in cultures challenged with anti-CD24 peptide antibody. A phospho-tyrosine profile characteristic of activated c-Src kinase confirmed results from inhibition of protein kinases, indicating that c-Src kinase mediated the modulation of epithelial monolayer barrier function.

CD24 embedded in membrane rafts is specifically recognized by the lectin siglec-10 [15]. Siglec-10 associates with SHP-1 tyrosine phosphatase which selectively activates c-Src kinase. This mechanism of signal transduction is further supported by evidence that

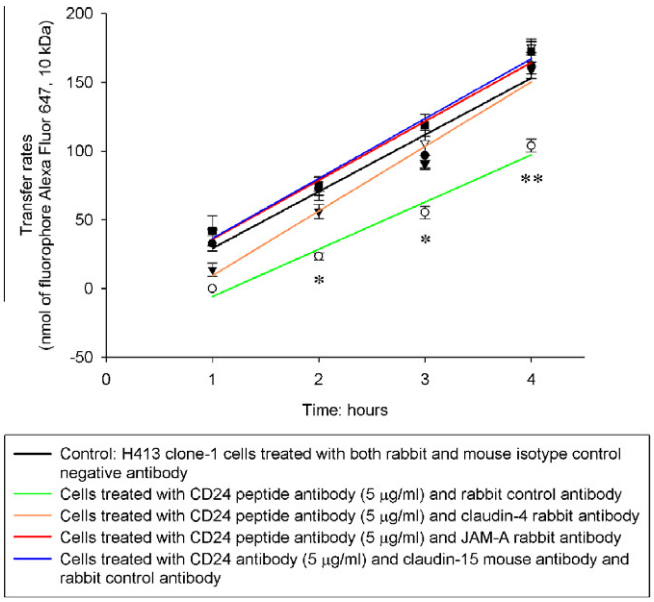

Fig. 4. Effect of antibodies specific for claudins-4, -15 and JAM-A on barrier function of anti-CD24-treated epithelial monolayers. As the passage of dextran into the lower compartment reached equilibrium after the 7th hour, four intermediate time points are shown to represent the rate/amount of transfer. Data show mean values ± S.D. for a representative of three independent experiments in triplicate for regression analysis of the effect of antibodies to claudins-4, -15 and JAM-A. Cultures were permeable for the translocation of low molecular weight dextran (Alexa Fluor 647 10 kDa, * refers to $^{*}P < 0.05$, $^{**}P < 0.01$, paired *t*-test) compared to CD24-treated epithelial monolayers.

Src kinases co-localize with affinity extracted CD24 [22]. A novel role for E-cadherin is suggested by the reported capacity of E-cadherin to bind and translocate c-Src kinase to the cell periphery in close proximity to organizing tight junctions [23]. Activation of c-Src kinase was not implicated in the synthesis of tight junction components as treatment with saracatinib did not affect enhanced expression levels in response to ligation of CD24. Previously we reported that ligation of CD24 on H413 epithelial cells was associated with up-regulation of *e-cadherin* expression [18]. The effect of ligation of CD24 on cell adhesion mechanisms was selective for E-cadherin and tight junction components as no effect was observed on the expression of genes encoding β-catenin, connexin-43 and connexin-26, integrin-β1, integrin-β6, integrin-αV or ICAM-1 [18]. Ligation of CD24 on H413 cells resulted in up-regulation of *par-3* and *-6* expression and peripheral reorganization of F-actin, changes typically associated with tight junction formation [16].

In the present study, tight junction components most implicated in mediating the barrier properties of H413 oral epithelial monolayers were discriminated on the basis of increase in gene expression and corresponding protein expression in response to ligation of CD24. This information was supplemented by confocal microscopy which demonstrated relocation of tight junction proteins to the cell periphery in response to ligation of CD24 and inhibition of this response by saracatinib. Only claudins-4 and -15 and JAM-A fulfilled all of these criteria. Co-incubation of H413 monolayers with specific antibodies reactive against surface-expressed tight junction components resulted in impaired barrier function, confirming the contribution of these entities to barrier function.

Claudin-4 has been implicated in the barrier function of stratified epithelia [24], while claudin-15 expression has recently been demonstrated to be indispensable for paracellular Na (+) permeability and efficient absorption of glucose in murine small intestine [25].

Junctional adhesion molecules (JAMs) belong to the immunoglobulin superfamily and are found at the tight junctions (TJs) of

polarized epithelial and endothelial cells. JAMs are characterized by a molecular structure composed of two extracellular immunoglobulin loops, a single transmembrane domain, and a cytoplasmic domain with a PDZ binding motif. JAMs interact with several ligands in both homophilic and heterophilic manner through the extracellular domain, and bind to various PDZ domain-containing proteins at the C-terminus. Through these protein–protein interactions, JAMs are implicated in diverse biological functions at TJs, including cell–cell adhesion, junctional assembly, junctional stabilization, regulation of paracellular permeability and leukocyte transmigration [26,27]. $JAM^{-/-}$ mice displayed increased mucosal permeability as indicated by enhanced dextran flux [28]. Evidence from the model system used in the present study indicated expression of JAM-A is also critical for increased barrier function against paracellular diffusion of low molecular weight bacterial products.

Uncertainties remain regarding the capacity of point contact maculae occludens, typical of stratified epithelia and observed in H413 epithelial monolayers following ligation of CD24, to effectively limit paracellular translocation of low molecular weight solutes [16]. Additional functions of tight junction components could contribute to barrier function [18]. Occludin is located exclusively at cell contacts following ligation of CD24 on confluent H413 monolayers [16]. Although occludin is detected as an integral 65 kDa tight junction strand protein [29], occludin-deficient mice have apparently normal epithelial function [30]. Occludin interacts directly or indirectly with claudins-1 and -2 to facilitate recruitment of these components into tight junction complexes [27]. An additional function is suggested by data indicating that occludin mediates the expulsion of apoptotic epithelial cells from cultured monolayers, thereby potentially preserving monolayer integrity [31].

In the model system, ligation of CD24 induced enhanced expression of both E-cadherin, critical in organizing tight junctions, and also selected expression of a number of tight junction components with claudin-15 and JAM-A particularly implicated in monolayer barrier function. Activation of c-Src kinase was linked to peripheral location of the tight junction components JAM-A, claudins-15 and -4 leading to enhanced barrier function. The findings extend knowledge of the control mechanisms for, and properties of, tight junction proteins, in this tissue location.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.07.067.

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