Antibody Blockade of Junctional Adhesion Molecule-A in Rabbit Corneal Endothelial Tight Junctions Produces Corneal Swelling

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Purpose. The ultrastructure of tight junctions in the corneal endothelium has been studied extensively, yet little is known about their molecular composition. Junctional adhesion molecule-A (JAM-A) is a tight junction–associated adhesion protein previously implicated in tight junction assembly and regulation of barrier function. In this study, we sought to investigate the expression and function of JAM-A in the corneal endothelium.

Methods. Immunofluorescence confocal microscopy was used to investigate expression of JAM-A and the related proteins JAM-C, CAR, and AF-6 in the rabbit corneal endothelium. Corneal endothelial perfusion specular microscopy was then used to test the effects of antibodies to JAM-A on corneal swelling.

Results. The expression of JAM-A was observed in the tight junctions of rabbit corneal endothelium in a localization pattern identical with that of ZO-1, a known marker of the tight junction and binding partner of JAM-A. Expression of related proteins JAM-C and CAR (Coxsackie and adenovirus receptor) was also observed in the corneal endothelium, but their distribution was diffuse and not limited to the tight junction. Expression of AF-6, a known binding partner of JAM-A, was also observed in the tight junction in a pattern similar to ZO-1. Last, functional experiments were performed in which a monoclonal antibody to JAM-A was shown to increase rabbit corneal swelling by 63% compared with the control.

Conclusions. The results provide new evidence that JAM-A and its binding partner AF-6 are expressed in tight junctions of the corneal endothelium and that JAM-A has a major role in maintaining the corneal endothelial barrier function. (*Invest Ophthalmol Vis Sci.* 2006;47:2408–2416) DOI:10.1167/iovs.05-0745

The corneal endothelium forms a physiologic barrier between the nutrient-rich aqueous humor and avascular corneal stroma, and tight junctions are an integral component of this barrier. In general, tight junctions serve to restrict paracellular diffusion of ions, water, and macromolecules. Although the junctions of the corneal endothelium are known to be "leaky, " their enhanced permeability permits diffusion of nutrients into the avascular stroma.[1] Along with nutrient diffusion into the stroma, Na/K-ATPases in the corneal endothelium actively transport salt out of the cornea, drawing water with it.[2] Together, this dynamic "pump–leak" system creates an osmotic gradient that maintains corneal deturgescence while permitting nutrient delivery into the stroma. Disruption of the endothelial barrier results in corneal edema and loss of optical transparency.

Junctional adhesion molecules (JAMs) are a family of adhesion molecules found in intercellular junctions.[3–6] Evidence suggests that JAMs are implicated in a variety of cellular process. For example, JAM-A is thought to be involved in regulation of tight junction permeability, leukocyte transmigration, angiogenesis, and platelet aggregation. In addition, evidence suggests that JAM-A is a receptor for reovirus, an enteric pathogen that can cause diarrhea in children.[7] JAM-C and the Coxsackie-adenovirus receptor (CAR) are closely related to JAM-A, and evidence suggests that these proteins have similar functions in cells, particularly with respect to leukocyte migration. One noted exception is that JAM-C is thought to be expressed exclusively in desmosomes, whereas JAM-A appears to be concentrated in tight junctions with some expression along the lateral membrane surface of polarized cells.

Structurally, JAM proteins are classified within the immunoglobulin superfamily (IgSF). The extracellular domain of JAMs contains two Ig-like loops, and evidence suggests that homophilic binding of JAMs via the N-terminal loop is important for its function in cells, particularly with respect to regulation of tight junction permeability.[8–11] Studies suggest that JAM dimers can self-associate to form homotetramers,[9] but it is also been suggested that JAMs interact heterophilically with other adhesion molecules such as integrins.[12,13] Such heterophilic interactions with integrins are thought to be most relevant to their role in leukocyte transmigration,[12,13] as opposed to their role in tight junctions, which is thought to be mediated by homophilic binding.[11]

In addition to extracellular interactions, JAMs contain a short cytoplasmic tail shown to mediate binding scaffold proteins of the postsynaptic density-95, discs large, and zonula occludens-1 (PDZ) family. JAM-A has been shown to interact specifically with tight junction-associated PDZ proteins that include zonula occludens (ZO)-1, the ALL-1 fusion partner from chromosome 6 (AF-6), calcium/calmodulin-dependent serine protein kinase (CASK), and atypical PKC isotype-specific interacting protein (ASIP).[14–16] Although the functional significance of such interactions is not well understood, evidence suggests that they serve as a link to signal transduction pathways critical for regulation of cell polarity, growth, and differentiation. For example, studies from our laboratory suggest that JAM-A expression regulates epithelial cell morphology, possibly by affecting of β1 integrins via the small GTPase Rap1.[17] We hypothesize that the link between JAM-A and the Rap1 pathway is mediated by a common interaction with AF-6 or a related PDZ protein.[18] Thus, the function of JAM proteins in the tight junction appears to involve not only extracellular adhesive interactions but also cytosolic interactions with PDZ

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proteins linked to signaling pathways that regulate cell growth and development.

In the corneal endothelium, the molecular composition of tight junctions is largely unknown. Although the ultrastructure of tight junctions in the corneal endothelium has been studied extensively,[1,19,20] little is known about the specific molecules that compose these structures. In fact, ZO-1 is the only tight junction protein to date that has been demonstrated in the corneal endothelium.[21] Although JAM-A has been implicated in tight junction function in variety of epithelial tissues,[3–5,8,11] no study to date has investigated the role of JAM proteins in the corneal endothelium. Our present study is therefore significant, because we demonstrate that JAM-A is expressed in tight junctions of the corneal endothelium along with one of its known binding partners, AF-6. In addition, we provide evidence that JAM-A regulates corneal endothelial barrier function as evidenced by the effect of JAM-A antibodies on corneal swelling after transient calcium-depletion. These results suggest that JAM-A is a functional component of corneal endothelial tight junctions, and we believe these findings provide a framework for further studies investigating the role of JAMs and their binding partners in the corneal endothelium.

METHODS

Antibodies

Monoclonal antibodies against the human JAM-A, clones 7G2C9, J3F.1, and 1H2A9 were raised in female BALBc mice immunized with recombinant human JAM-A protein according to previously described methods.[3] Immunoreactivity of 7G2C9, J3F.1, and 1H2A9 with JAM-A was confirmed by ELISA and by immunofluorescence staining of T84 epithelial cell monolayers. Polyclonal antibody against JAM-A was raised in rabbits using the recombinant extracellular domain of human JAM-A as the antigen.[3] Mouse monoclonal antibody against human JAM-C (clone MAB1189) was obtained from R&D Systems (Minneapolis, MN). Mouse monoclonal antibody against human CAR (clone RmcB) was obtained from Upstate Biotechnology (Lake Placid, NY). Mouse monoclonal antibody against human ZO-1 (clone ZO-1-1A12) was obtained from Zymed Laboratories, Inc. (South San Francisco, CA). Mouse monoclonal antibody against human AF-6 (clone 35) was obtained from Transduction Laboratories (San Jose, CA). Alexa Fluor 488-conjugated goat anti-mouse antibodies were obtained from Invitrogen (Eugene, OR).

Confocal Immunofluorescence Microscopy

New Zealand White rabbits were killed, and their corneas were isolated and fixed in 10% acetic acid in 100% ethanol at −20°C for 20 minutes. Fixed corneas were blocked in 1% bovine serum albumin in Hanks' balanced salt solution containing calcium and magnesium ($HBSS^+$). Primary antibodies to JAM-A, JAM-C, CAR, ZO-1, and AF-6 were diluted in blocking buffer at a concentration of 2 μg/mL and incubated with corneas for 1 hour at room temperature. Primary-labeled corneas were washed twice in $HBSS^+$ and then labeled with secondary antibodies for 1 hour at room temperature. Nuclei were counterstained with To-Pro 3 nucleic acid stain (Invitrogen), washed twice with HBSS+, and mounted (Prolong Antifade Agent; Invitrogen). Immunofluorescence confocal microscopy was performed with a laser scanning microscope (LSM 510; Carl Zeiss Meditec, Inc., Oberkochen, Germany). For competitive binding experiments, the primary antibody 7G2C9 (2 μg/mL) was incubated with a 10-fold excess of recombinant soluble JAM-A (20 μg/mL) for 1 hour at room temperature. The remainder of the protocol was performed as described earlier. All experiments were conducted in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Institutional Animal Care and Use Committee of Emory University.

Western Blot Analysis

Intact rabbit corneal endothelium was dissected from whole rabbit corneas obtained from Pel-Freez (Rogers, AR) and detached enzymatically from Descemet's membrane by treatment with 0.05% trypsin/EDTA for 20 minutes at 37°C. Endothelial cells were collected by centrifugation and lysed in buffer containing 20 mM Tris, 50 mM NaCl, 2 mM EDTA, 2 mM EGTA, 1% sodium deoxycholate, 1% Triton X-100, and 0.1% SDS (pH 7.4). Lysis buffer was supplemented with protease and phosphatase inhibitor cocktails containing AEBSF, pepstatinA, E-64, bestatin, leupeptin, aprotinin, microcystin LR, cantharidin, (−)-*p*-bromotetramisole, sodium vanadate, sodium molybdate, sodium tartrate, and imidazole (1:100 dilution; Sigma-Aldrich). Lysates were then cleared by centrifugation and immediately boiled in reduced SDS sample buffer. SDS-PAGE and immunoblots were performed by standard methods. The anti-JAM-A monoclonal antibody 7G2C9 was used at a concentration of 5 μg/mL, and the anti-ZO-1 monoclonal antibody (Zymed) was used at 2 μg/mL.

Tight Junction Permeability Assays

Transepithelial resistance (TER) to passive ion flow was monitored in epithelial cell monolayers using a calcium-switch method, as previously reported.[3,11] In brief, SK-CO15 epithelial cells were grown on permeable polycarbonate membranes (0.4-μm pore size). TER values were recorded using a voltmeter (EVOMX, with an STX2 electrode; World Precision Instruments, Sarasota, FL). Initial resistance values for confluent SK-CO15 monolayers 4 to 7 days after seeding were consistently greater than 1000 $\Omega \cdot cm^2$. To disrupt intercellular junctions, cells were treated with calcium-free medium (minimum essential medium with 10 mM HEPES, 18 mM $NaHCO_3$, 6% newborn calf serum dialyzed in phosphate-buffered saline, and 2 mM EGTA). After 45 minutes at 37°C in calcium-free medium, TER was recorded for each monolayer yielding TER values less than 50 $\Omega \cdot cm^2$. Cells were washed twice with normal growth medium and incubated in normal growth medium containing 10 μg/mL of antibody anti-JAM-A mAb 7G2C9 or an isotype-matched IgG control at 37°C. Sequential TER measurements were made at various time points thereafter. TER recovery was reported as the percentage of the final TER relative to the initial TER value for each monolayer.

Corneal Swelling Assays

Assays for corneal swelling were performed as previously reported.[22] In brief, New Zealand White rabbits were killed, and corneas were isolated and mounted in pairs in an in vitro specular microscope. Corneal endothelia were perfused in glutathione bicarbonated Ringer's (GBR) solution containing 6.521 g/L NaCl, 0.358 g/L KCl, 0.115 g/L $CaCl_2$, 0.159 g/L $MgCl_2$, 0.103 g/L NaH_2PO_4, 2.454 g/L $NaHCO_3$, 0.9 g/L glucose, and 0.92 g/L reduced glutathione. Temperature and pressure were maintained at 37°C and 15 mm Hg. After perfusion in GBR for 15 minutes, corneas were switched to calcium-free GBR for 1 hour, to disrupt cell junctions and induce corneal swelling. After 1 hour, corneas were switched back to either GBR alone for controls or to GBR containing anti-JAM-A antibody 7G2C9 (10 μg/mL). Changes in corneal thickness were then measured at various time points thereafter. For each corneal thickness measurement, the specular microscope was focused on the corneal epithelium, and the position of the focal plane of corneal epithelium was recorded with a digital readout linked to the focus adjustment mechanism. A second recording was then made at the focal plane corresponding to the corneal endothelium, and corneal thickness was calculated in micrometers as the distance between the epithelial and endothelial layers. Corneal swelling was reported as the change in corneal thickness relative to the initial value for each cornea at the time that the antibody was added. Reported values represent the average of three independent experiments with probabilities calculated using a Students *t*-test for paired samples.

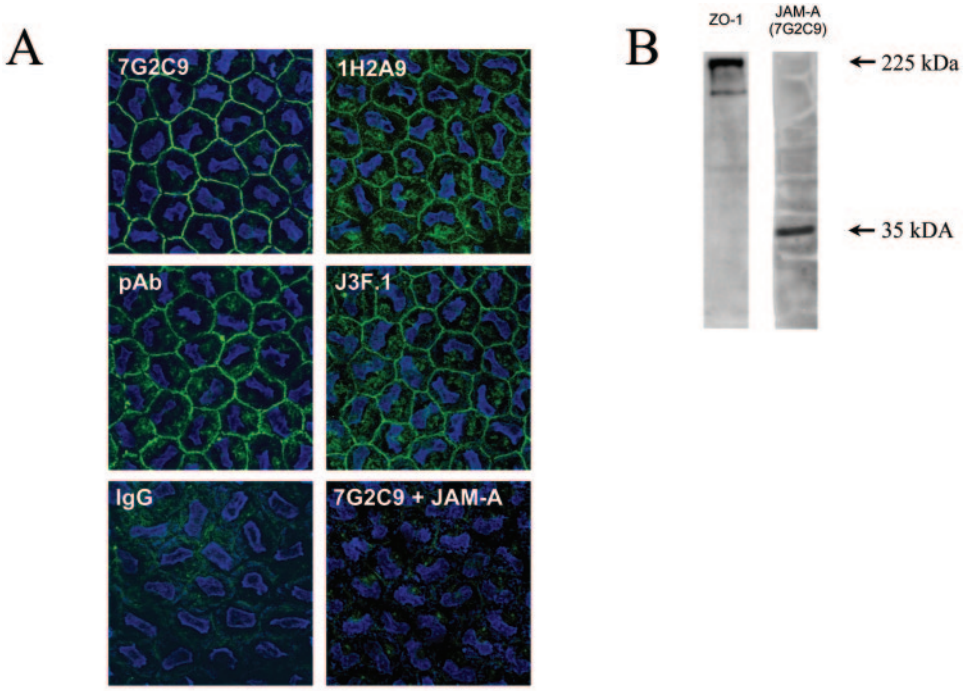

FIGURE 1. The monoclonal antibody 7G2C9 reacted specifically with JAM-A in rabbit corneal endothelium. (**A**) Immunofluorescence confocal microscopy was performed in rabbit corneal endothelium to evaluate the immunoreactivity of various JAM-A antibodies. 7G2C9, J3F.1, and 1H2A9 are mouse-derived monoclonal antibodies raised against recombinant human JAM-A.[3,11] pAb is a rabbit-derived polyclonal antibody raised against recombinant human JAM-A.[3] IgG is an isotype-matched control antibody derived from the mouse. 7G2C9+JAM-A is the monoclonal antibody 7G2C9, with a 10-fold excess of recombinant JAM-A antigen. (**B**) Western blot analyses were performed to assess the specificity of the monoclonal antibody 7G2C9 for JAM-A in rabbit corneal endothelial cells. Note that the 7G2C9 antibody detects a single band at ~35 kDa, which is the predicted size of JAM-A. The 225-kDa tight junction protein ZO-1 is shown as a control.

RESULTS

Reaction between Monoclonal Antibody 7G2C9 and JAM-A in the Rabbit Corneal Endothelium

JAM-A is a tight junction–associated adhesion molecule known to be expressed in tight junctions in various tissues. In this study, we sought to investigate whether JAM-A is expressed in tight junctions of the rabbit corneal endothelium. Although anti-JAM-A monoclonal antibodies have been used extensively to investigate JAM-A expression and function in human, murine, and canine systems, no such studies have been performed in the rabbit. Having previously developed a large panel of antibodies for study of human JAM-A, we therefore sought to screen for specific clones that react with rabbit JAM-A. To do so, immunofluorescence confocal microscopy was performed with various anti-JAM-A antibodies in rabbit corneal endothelium. As shown in Figure 1A, monoclonal antibodies 1H2A9 and J3F.1, which are known to stain tight junctions in human tissue,[3,11] were observed to react with rabbit corneal endothelial junctions with moderate intensity but with some nonspecific background staining as well. Likewise, a polyclonal antibody raised against human JAM-A was also observed to react modestly with rabbit corneal endothelial junctions, but it too produced some degree of background staining (Fig. 1A). In contrast to these antibodies, one specific clone, 7G2C9, produced very discrete junctional staining without significant background (Fig. 1A). To address the possibility that binding of this antibody is due to nonspecific Fc-mediated interactions, binding of an isotype-matched control Ig was tested. As shown in Figure 1A, no staining was observed for the IgG control. To further confirm that antibody 7G2C9 reacts specifically with JAM-A in cell junctions, a 10-fold excess of recombinant JAM-A was added. As shown in Figure 1A, treatment with excess recombinant JAM-A completely abolished staining of 7G2C9 in rabbit corneal endothelial junctions. Taken together, these results suggest that monoclonal antibody 7G2C9 specifically detects JAM-A in rabbit corneal endothelial junctions.

In addition to assessing the specificity of antibody 7G2C9 by immunofluorescence microscopy, Western blot analyses were performed with lysate from rabbit corneal endothelial cells. As shown in Figure 1B, Western blot analysis with antibody 7G2C9 produced a single band at ~35 kDa, the predicted size for JAM-A. As a positive control, a Western blot for the tight junction-associated protein ZO-1 was also performed, and a single band at ~225 kDa was observed. Together with the immunofluorescence data, the Western blot experiments demonstrate that JAM-A is expressed in intercellular junctions of the rabbit corneal endothelium and monoclonal antibody 7G2C9 can specifically detect expression of this protein.

JAM-A in Tight Junctions of the Rabbit Corneal Endothelium

Having observed that JAM-A is expressed in rabbit corneal endothelial cell junctions, we conducted further experiments to assess its subcellular localization. Fresh rabbit corneas were fixed and labeled with monoclonal antibody 7G2C9, and localization of JAM-A was assessed by immunofluorescence confocal microscopy. As shown in the en face images in Figure 2A, JAM-A was observed to localize at cell–cell junctions. In addition, the transverse cross-section shown in Figure 2C illustrates the presence of JAM-A at the apical-most portion of the lateral cell membrane in the region of the tight junction. Comparison with the tight junction marker ZO-1 revealed that JAM-A and ZO-1 exhibit nearly identical localization patterns in the rabbit corneal endothelium (Figs. 2B, 2D). In fact, examination of JAM-A localization at higher magnifications (Fig. 3) revealed discontinuities at Y-junctions where three cells intersect. These gaps in JAM-A expression are similar to those observed for ZO-1 in other studies, and such discontinuities in the TJ may explain the relatively low resistance of corneal endothelium.[21,23,24] Overall, our results provide evidence that JAM-A is expressed in tight junctions of the corneal endothelium, and its distribution pattern is consistent with the known tight junction marker ZO-1.

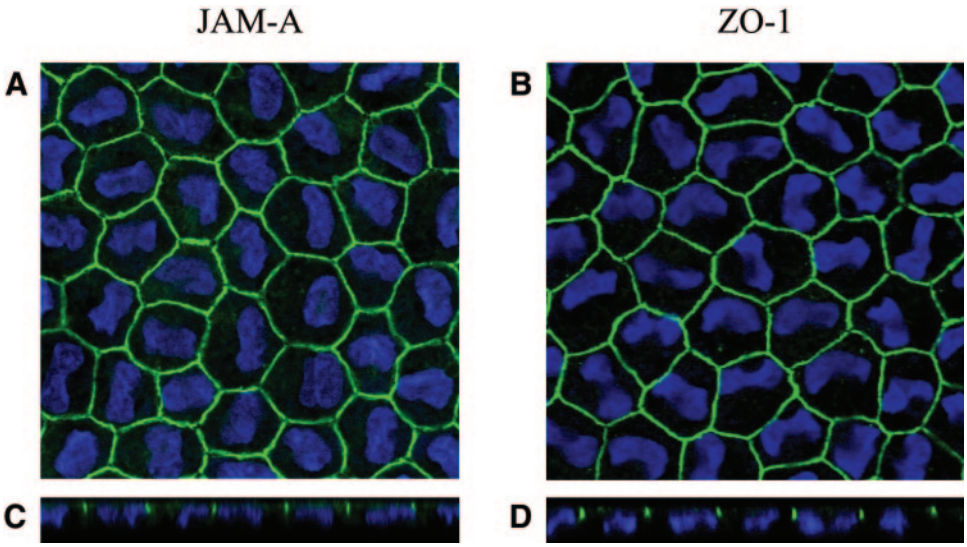

FIGURE 2. Localization of JAM-A in tight junctions of the corneal endothelium. Expression of JAM-A in rabbit corneal endothelium was assessed by immunofluorescence confocal microscopy and compared with the tight junction marker ZO-1. En face images (**A**, **C**) show expression of JAM-A and ZO-1 at cell–cell junctions (*green*). Confocal cross-sections (**B**, **D**) of the same specimens illustrate localization of JAM-A and ZO-1 in the apical-most part of the lateral cell membrane. Nuclei are counterstained *blue*.

Expression of JAM-C and CAR in the Rabbit Corneal Endothelium

Having observed the expression of JAM-A in the corneal endothelium, we performed experiments to investigate whether the related proteins JAM-C and CAR were also expressed. Fresh rabbit corneas were fixed and labeled with antibodies against JAM-C and CAR and expression of JAM-C and CAR was assessed by immunofluorescence confocal microscopy. As illustrated in Figure 4, expression of JAM-C and CAR was observed in corneal endothelium, but their localization was less discrete than that of JAM-A and was not limited to intercellular junctions. Although JAM-A was seen exclusively in tight junctions of endothelial cells (Fig. 2), staining for JAM-C and CAR was more diffuse and mottled in appearance, with some expression apparent on the apical surface (Fig. 4). These results suggest that JAM-C and CAR are expressed in the corneal endothelium, but they do not exhibit the discrete tight junction-associated staining pattern seen with JAM-A. Such differences in subcellular localization may simply reflect differences in the function of JAM-C and CAR compared to JAM-A in the corneal endothelium. For example, in intestinal epithelial cells, JAM-C has been observed to localize in desmosomes rather than tight junctions, and its function appears to relate to leukocyte migration rather than paracellular permeability.[25]

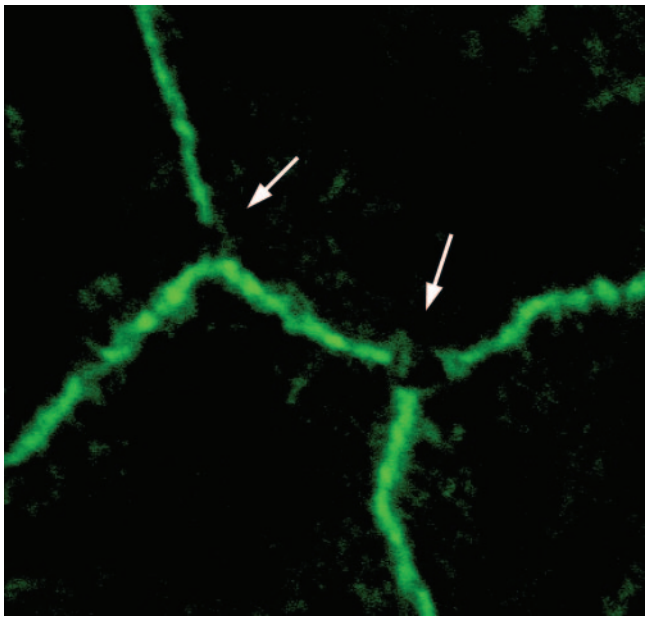

FIGURE 3. High-magnification images of JAM-A expression in corneal endothelial junctions. Immunofluorescence confocal micrographs demonstrate gaps in JAM-A expression at Y-junctions where three endothelial cells meet (*arrows*). This pattern is consistent with the study by Barry et al.[21] demonstrating similar gaps in ZO-1 at endothelial Y-junctions.

AF-6 in Tight Junctions of the Rabbit Corneal Endothelium

PDZ scaffold proteins such ZO-1 and AF-6 play a vital role in organizing the numerous transmembrane and cytosolic proteins that comprise the tight junction. JAM-A is known to bind to both ZO-1 and AF-6 through its PDZ-binding domain.[15,26] In addition, evidence suggests that interactions between JAM-A and AF-6 underlie JAM-A-mediated regulation of cell morphology via the Rap1 pathway.[17] Given these known relationships between JAM-A and AF-6, we sought in investigate whether AF-6 is expressed in tight junctions of the corneal endothelium. Fresh rabbit corneas were fixed and labeled with antibodies against AF-6, and immunofluorescence confocal microscopy was performed. As shown in Figure 5A, expression of AF-6 was observed in intercellular junctions of the corneal endothelium. The transverse cross-sectional images shown in Figure 5B illustrate that, like JAM-A and ZO-1, AF-6 localizes in the apicalmost part of the lateral cell membrane in the region of the tight junction. Overall, our results strongly suggest that AF-6 is a component of corneal endothelial tight junctions, and this is consistent with previous studies that report a functional relationship between AF-6 and JAM-A.[15,17]

Effect of Antibodies to JAM-A on Swelling of Rabbit Corneas

Antibodies to JAM-A have been shown to disrupt tight junctions and increase paracellular permeability across cell monolayers in vitro.[3,4,11] Given our observation that JAM-A is expressed in corneal endothelial tight junctions (Fig. 1), we sought to investigate whether an antibody to JAM-A (clone 7G2C9) can affect the permeability of the corneal endothelium. Before performing experiments with this antibody in whole rabbit corneas, however, we first sought to confirm that

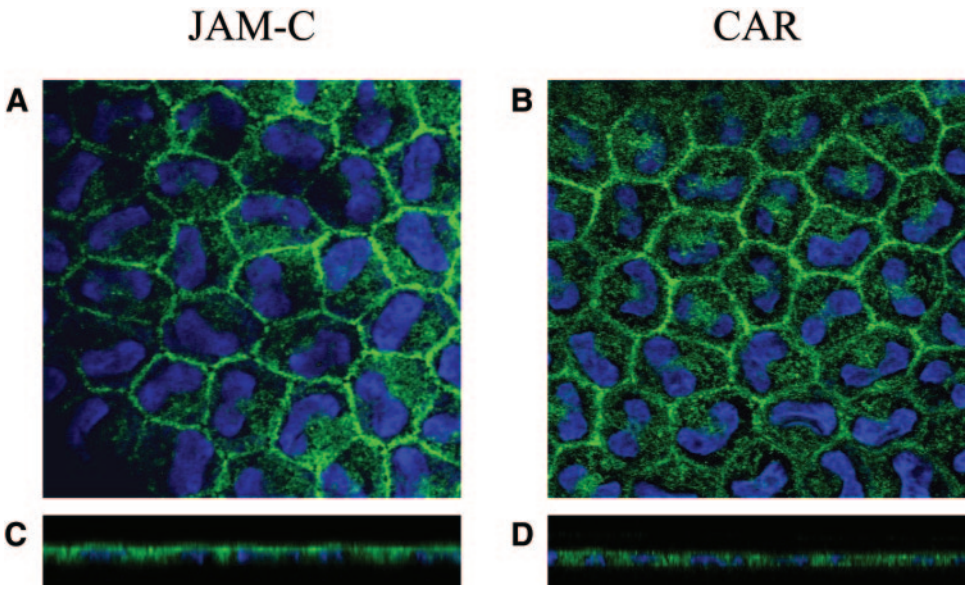

FIGURE 4. Expression of JAM-C and CAR in the corneal endothelium. Expression of JAM-C and CAR in rabbit corneal endothelium was assessed by immunofluorescence confocal microscopy. En face images (**A**, **C**) show junctional expression of JAM-C and CAR with diffuse extrajunctional expression as well (*green*). Confocal cross-sections (**B**, **D**) of the same specimens show diffuse expression of JAM-C and CAR. Nuclei are counterstained *blue*.

our antibody has functional effects on cell monolayers in vitro. To do so, a "calcium-switch" protocol was performed as previously described.[3,11] As shown in Figure 6A, TER recovery was monitored at a series of time points after calcium-repletion in the presence of antibody to JAM-A or an isotype-matched control. After 10 hours of incubation, control cells recovered more than 60% of their initial TER, whereas JAM-A antibody-treated cells recovered to only 12% (Fig. 6A). This effect of JAM-A antibody on TER was observed to be statistically significant at 2.5, 6, and 10 hours ($P < 0.05$).

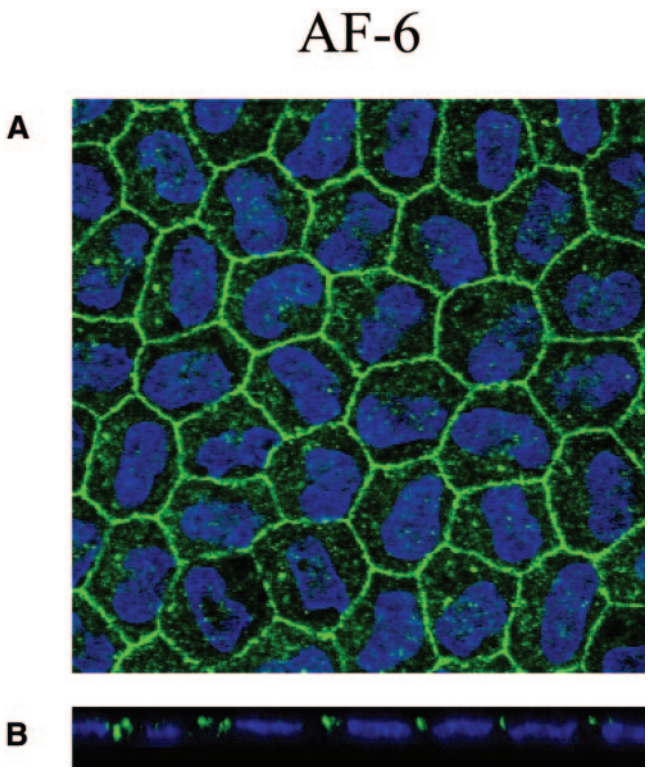

FIGURE 5. Localization of AF-6 in tight junctions of the corneal endothelium. Expression of AF-6 in rabbit corneal endothelium was assessed by immunofluorescence confocal microscopy. The en face image (**A**) shows expression of AF-6 at cell–cell junctions (*green*). The confocal cross-section (**B**) of the same specimen shows localization of AF-6 in the apicalmost part of the lateral cell membrane. Nuclei are counterstained *blue*.

Having confirmed that our JAM-A antibody affects cell monolayer permeability in vitro, we then performed experiments to determine the effect of JAM-A antibody on rabbit corneal endothelium. Fresh corneas from New Zealand White rabbits were mounted in pairs in an in vitro specular microscope, and the endothelial surfaces were perfused with GBR solution. Corneas were transiently perfused in calcium-free GBR to initiate corneal swelling and then switched back to complete GBR in the presence or absence of JAM-A antibody. Corneal swelling was measured in terms of changes in corneal thickness, as assessed by measurement in the specular microscope at various time points after addition of the anti-JAM-A antibody. As shown in Figure 6B, both the control corneas and corneas treated with JAM-A antibody continued to swell after calcium restitution, but the JAM-A antibody-treated corneas swelled to a much greater degree. After 160 minutes, the average swelling of control corneas was 51 μm versus 83 μm in JAM-A antibody-treated corneas (Fig. 6B). In total, treatment with the JAM-A antibody produced a 63% increase in swelling relative to the control, and this difference was statistically significant ($P < 0.05$). Overall, these results show that treatment of the endothelium with a JAM-A antibody dramatically affected swelling of the rabbit cornea. This functional effect is consistent with JAM-A's being a component of corneal endothelial tight junctions (Fig. 2) and supports previous studies suggesting that JAM-A regulates tight junction permeability.[3,4,11,17]

DISCUSSION

Tight junctions play an important role in maintaining the integrity of the corneal endothelial barrier. Although corneal endothelial tight junctions have been studied extensively at the ultrastructural level, little is known about their molecular composition. Before this study, the only protein known to be expressed in corneal endothelial tight junctions was the PDZ scaffold protein ZO-1.[21] In addition, none of the canonical transmembrane adhesion molecules normally found in tight junctions, be it claudins, occludins or JAMs, had been identified in corneal endothelium. Thus, our present findings are significant, providing evidence that JAM-A, a tight junction–

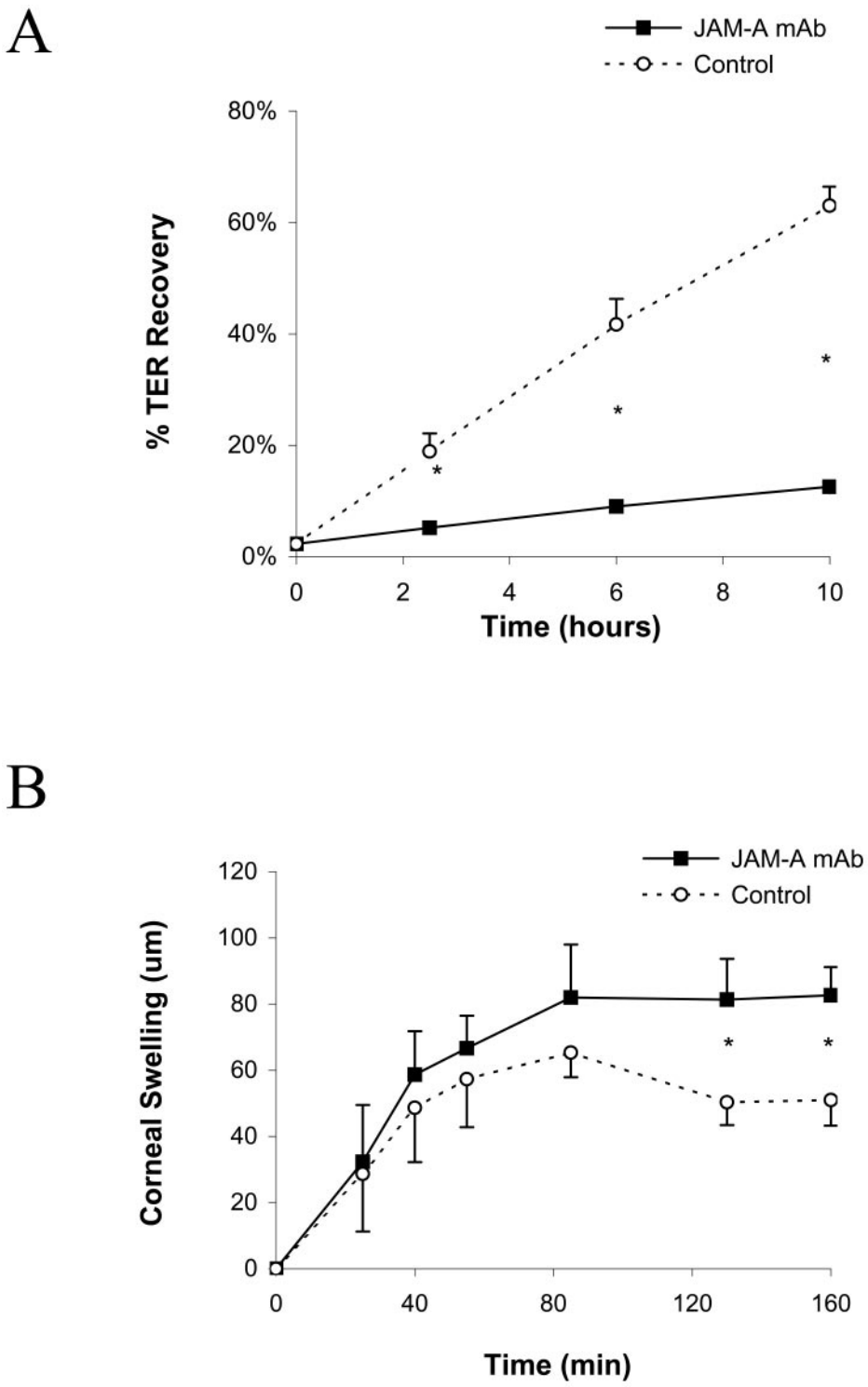

FIGURE 6. An antibody against JAM-A increases TJ permeability and corneal swelling. (**A**) The effect of the anti-JAM-A antibody 7G2C9 on TER recovery after transient calcium depletion. (**B**) The effect of anti-JAM-A antibody 7G2C9 on rabbit cornea swelling after transient calcium depletion. Error bars, SEM; $^*P < 0.05$.

associated adhesion molecule, and AF-6, a tight junction–associated PDZ protein and binding partner of JAM-A, are expressed in tight junctions of the corneal endothelium. In addition to demonstrating expression of these proteins, we provide functional data showing that JAM-A plays a role in regulation of corneal swelling. Our findings provide insight into the molecular composition of corneal endothelial tight junctions and suggest that JAM-A and AF-6 may be useful targets for future study of the corneal endothelial barrier in various corneal diseases.

JAM proteins are expressed in a variety of types of cells throughout the human body. JAM-A has a particularly broad expression profile that includes epithelial cells, endothelial cells, platelets, and leukocytes.[3–5,27–29] In tall columnar epithelial cells, JAM-A has been observed primarily at the apicalmost part of the lateral membrane in the region of the tight junction.[3] To a lesser extent, JAM-A is also observed along the lateral membrane of epithelial cells, and the functional significance of these two pools is not known.[3] Given that corneal endothelial cells are basically squamous epithelium, it is not

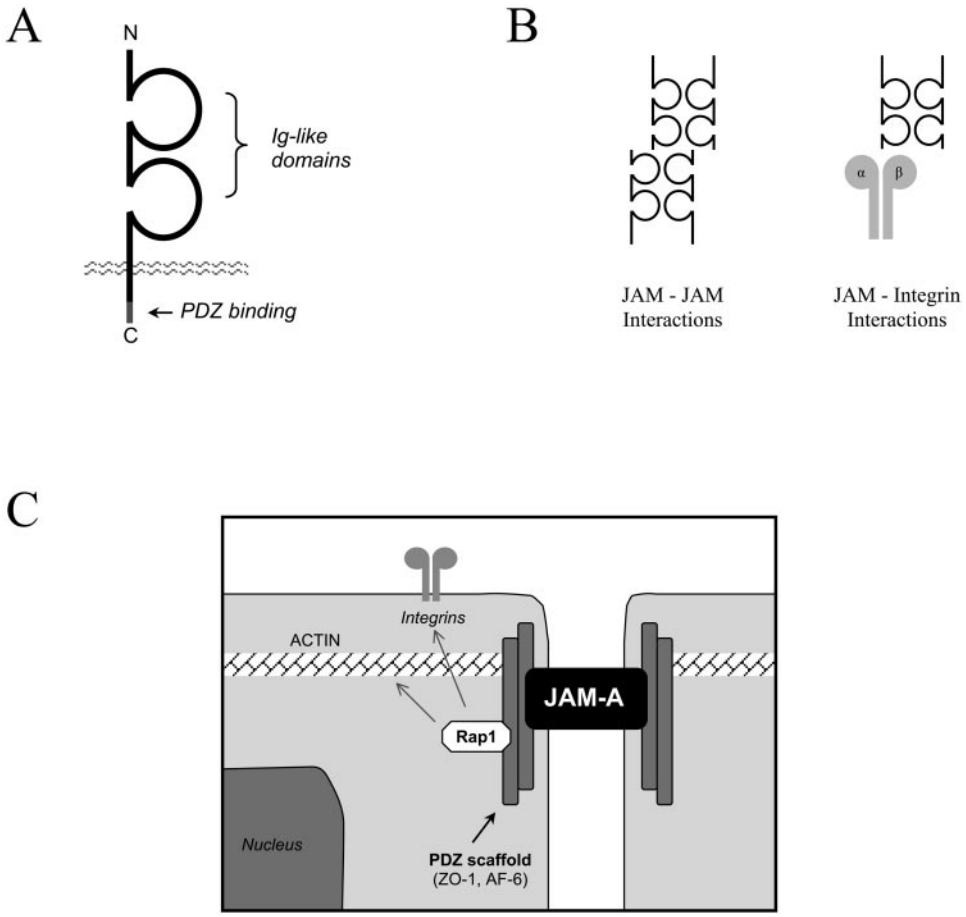

FIGURE 7. Junctional adhesion molecules and their role in tight junctions. (**A**) The structure of JAMs: two extracellular Ig-like loops, a single membrane-spanning domain, and short cytoplasmic tail (~40 amino acids) terminating in the PDZ-binding domain. (**B**) Self association of JAMs results in dimers and homo- and heterotetramers.[8–11,12,13,25] (**C**) Interactions between JAM-A and PDZ scaffold proteins such as ZO-1 and AF-6 are thought to regulate cell polarity via small GTPases such as Cdc42 and Rap1.[15,17] These pathways may ultimately affect cell morphology through changes in β1 integrin expression and the actin cytoskeleton.

surprising that their tight junctions would contain JAM-A, just like other epithelial tissues. It is noteworthy, however, that the JAM-A was observed exclusively in the tight junction of the corneal endothelium (Fig. 2) in contrast to tall columnar epithelial cells, where lateral membrane staining was also seen. Although this observed difference may reflect actual differences in the functions of JAM-A in these tissues, it is also possible that the antibody used to detect JAM-A in rabbit simply recognizes a subset of the JAM-A epitopes present in cells. This possibility is especially relevant, considering that the antibody used to detect JAM-A in this study, clone 7G2C9, was raised against human JAM-A protein and not the rabbit homologue. Yet, based on its ability to stain rabbit endothelium, antibody 7G2C9 clearly reacts with high specificity to the rabbit form of JAM-A (Fig. 1) and has functional effects in rabbit corneal endothelium (Fig. 6). These findings alone suggest that JAM-A is a highly conserved adhesion molecule whose expression has been evolutionarily conserved in a broad range of tissues among distantly related mammals.

Functionally, tight junctions of the corneal endothelium are known to be "leaky" compared with those of columnar epithelial cells, and studies suggest that this leakiness may in part be due to discontinuities at Y-junctions where three corneal endothelial cells meet.[21,23,24] Gaps in ZO-1 staining, for example, have observed such Y-junctions,[21] and it was expected that such discontinuities would be present for JAM-A as well. To investigate this possibility, we performed high-magnification confocal microscopy with antibody 7G2C9, and as with ZO-1, gaps in JAM-A expression were seen specifically at Y-junctions (Fig. 3). Together, these results provide further evidence that the leakiness of the corneal endothelium may be in part be due to gaps in the structure of tight junctions.

In addition to investigating expression of JAM-A, experiments were performed to investigate the expression of its close relatives JAM-C and CAR. In contrast to JAM-A, which appeared to be expressed exclusively in tight junctions of the corneal endothelium, expression of JAM-C and CAR was more diffuse and not limited to intercellular junctions (Fig. 4). It is possible that these observed differences reflect actual differences in function of JAM-C and CAR in the corneal endothelium. For example, JAM-C has been reported to be a component of desmosomes rather than tight junctions, and its function appears to be more related to leukocyte transmigration than to regulation of paracellular permeability.[25] It should be noted, however, that the observed differences in staining for JAM-C and CAR may also be explained by variability in the fixation and immunolabeling protocols used in this study. Like the JAM-A antibody, the JAM-C and CAR antibodies were raised against human proteins, and fixation and immunolabeling procedures were optimized in human epithelial cell lines. It is therefore conceivable that the antibodies used to stain rabbit tissue do not react optimally with the rabbit homologues of JAM-C and CAR or they cross-react with other epitopes found in the rabbit corneal endothelium. Further studies are needed to characterize thoroughly the subcellular localization of JAM-C and CAR in the corneal endothelium.

The mechanism by which JAM-A affects the TJ barrier function is not completely understood. As shown in the model presented in Figure 7, evidence suggests that the N-terminal Ig domain of JAM-A mediates homophilic binding that is important for JAM-A function in tight junctions. Studies have also shown that JAM proteins bind heterophilically to integrins, and these JAM-integrin interactions may be more relevant to leukocyte migration. In addition to extracellular adhesive interac-

tions, the cytosolic domain of JAMs are known to bind a host of PDZ scaffold proteins found in intercellular junctions, including ZO-1, AF-6, ASIP, and CASK.[14–16] These PDZ proteins play an integral role in the assembly and stabilization of cell junctions, presumably through interactions with adhesion molecules, the actin cytoskeleton and signal transduction pathways that regulate cell growth and development (Fig. 7C). For example, AF-6 is known to bind to both JAM-A[15] and the small GTPase Rap1,[18] and evidence suggests that expression of JAM-A regulates cell morphology via activation of Rap1.[17] In our present study, we demonstrate AF-6 expression in tight junctions of the corneal endothelium (Fig. 5), but further studies are needed to elucidate the precise relationship between JAM-A and AF-6 in the corneal endothelium and its affect on the corneal endothelial barrier function.

Evidence suggests that JAM-A has an important role in the regulation of tight junction permeability.[3,4,11] Antibodies to JAM-A and knockdown of JAM-A by small interfering RNA (si)RNA have both been shown to reduce dramatically the TER to passive ion flux in cell monolayers.[3,4,11,17] In the present study, we extended these observations to the corneal endothelium by showing that an antibody to JAM-A dramatically increases corneal swelling after transient calcium depletion. As shown in Figure 6B, treatment of rabbit corneal endothelium with antibody to JAM-A resulted in a 63% increase in swelling compared with the control. The kinetics of swelling suggest that both the control and antibody-treated groups continued to swell after calcium was repleted, but the JAM-A antibody appeared to swell to a much greater degree (Fig. 6B). This observation is understandable, because calcium-depletion is known to disrupt intercellular junctions reversibly, and recovery from calcium depletion is not an instantaneous process. The data from the endothelial-perfused corneas also suggest that the antibodies to JAM-A disrupted or delayed junctional resealing after the calcium switch, because these corneas continued to swell. Although antibody-antigen interactions tend to have a high affinity, it is likely that the function-blocking JAM-A antibody was eventually degraded, endocytosed, or outcompeted by endogenous ligand, thereby allowing junctional resealing to continue. Further studies, however, are needed to determine the exact mechanisms by which the JAM-A antibodies affect corneal swelling.

It is interesting that the time course of the effect of anti-JAM-A antibody on corneal swelling differed from that of TER recovery. That is, the TER recovery process occurred over the course of many hours (Fig. 6A), whereas the time course of corneal swelling was on the order of minutes to hours (Fig. 6B). One potential explanation is that there are inherent differences in morphology and functional properties of the cells assayed in these two experiments. In the case of the TER recovery assay, tall columnar epithelial cells with high-resistance tight junctions ($>1500\ \Omega \cdot cm^2$) were used. In contrast, the corneal swelling assays were performed on whole rabbit corneas, with the squamous endothelium having relatively low-resistance junctions ($<100\ \Omega \cdot cm^2$). It is therefore possible that the basic kinetics of junction assembly and disassembly differ among these cells types, and consequently the time it takes for the TJ barrier to recover in these cells may differ as well. A second possibility is that the mechanism by which the anti-JAM-A antibody affects corneal swelling may not be directly related to TER and TJ permeability. For example, TJ proteins are also thought to play an important role in signal transduction and regulation of cell polarity. Antibody 7G2C9 affect the polarity of the endothelial cells, thereby interfering with other proteins critical for endothelial deturgescence, such as the Na/K-ATPase pumps.

Overall, the data presented in this study provide evidence that JAM-A and its putative binding partner AF-6 are expressed in cell junctions of the corneal endothelium. In addition, it appears that JAM-A-related proteins such as JAM-C and CAR are also expressed in the corneal endothelium. Although the functional properties of JAM-A are not entirely understood at this time, evidence presented in this study suggests that JAM-A plays a role in regulation of corneal swelling. Further studies are needed to elucidate the mechanism by which this occurs.

References

1. Fromm M, Levarlet B, Kreusel KM, Hirsch M. Tight junctions of the human corneal endothelium: morphological and electrophysiological features. *Ger J Ophthalmol.* 1994;3:253–257.
2. Geroski DH, Edelhauser HF. Quantitation of Na/K ATPase pump sites in the rabbit corneal endothelium. *Invest Ophthalmol Vis Sci*. 1984;25:1056–1060.
3. Liu Y, Nusrat A, Schnell FJ, et al. Human junction adhesion molecule regulates tight junction resealing in epithelia. *J Cell Sci.* 2000; 113;2363–2374.
4. Liang TW, DeMarco RA, Mrsny RJ, et al. Characterization of huJAM: evidence for involvement in cell-cell contact and tight junction regulation. *Am J Physiol.* 2000;279:C1733–C1743.
5. Martin-Padura I, Lostaglio S, Schneemann M, et al. Junctional adhesion molecule, a novel member of the immunoglobulin superfamily that distributes at intercellular junctions and modulates monocyte transmigration. *J Cell Biol*. 1998;142:117–127.
6. Mandell KJ, Parkos CA. The JAM family of proteins. *Adv Drug Deliv Rev*. 2005;57:857–867.
7. Barton ES, Forrest JC, Connolly JL, et al. Junction adhesion molecule is a receptor for reovirus. *Cell*. 2001;104:441–451.
8. Bazzoni G, Martinez-Estrada OM, Mueller F, et al. Homophilic interaction of junctional adhesion molecule. *J Biol Chem*. 2000; 275:30970–30976.
9. Kostrewa D, Brockhaus M, D'Arcy A, et al. X-ray structure of junctional adhesion molecule: structural basis for homophilic adhesion via a novel dimerization motif. *EMBO J*. 2001;20:4391–4398.
10. Prota AE, Campbell JA, Schelling P, et al. Crystal structure of human junctional adhesion molecule 1: implications for reovirus binding. *Proc Natl Acad Sci USA*. 2003;100:5366–5371.
11. Mandell KJ, McCall IC, Parkos CA. Involvement of the junctional adhesion molecule-1 (JAM1) homodimer interface in regulation of epithelial barrier function. *J Biol Chem*. 2004;279:16254–16262.
12. Cunningham SA, Rodriguez JM, Arrate MP, Tran TM, Brock TA. JAM2 interacts with alpha4beta1: facilitation by JAM3. *J Biol Chem*. 2002;277:27589–27592.
13. Ostermann G, Weber KS, Zernecke A, Schroder A, Weber C. JAM-1 is a ligand of the beta(2) integrin LFA-1 involved in transendothelial migration of leukocytes. *Nat Immunol*. 2002;3:151–158.
14. Martinez-Estrada OM, Villa A, Breviario F, Orsenigo F, Dejana E, Bazzoni G. Association of junctional adhesion molecule with calcium/calmodulin-dependent serine protein kinase (CASK/LIN-2) in human epithelial caco-2 cells. *J Biol Chem*. 2001;276:9291–9296.
15. Ebnet K, Schulz CU, Meyer Zu Brickwedde MK, Pendl GG, Vestweber D. Junctional adhesion molecule interacts with the PDZ domain-containing proteins AF-6 and ZO-1. *J Biol Chem*. 2000;275: 27979–27988.
16. Ebnet K, Suzuki A, Horikoshi Y, et al. The cell polarity protein ASIP/PAR-3 directly associates with junctional adhesion molecule (JAM). *EMBO J*. 2001;20:3738–3748.
17. Mandell KJ, Babbin BA, Nusrat A, Parkos CA. Junctional adhesion molecule 1 regulates epithelial cell morphology through effects on beta1 integrins and Rap1 activity. *J Biol Chem*. 2005;280:11665–11674.
18. Boettner B, Govek EE, Cross J, Van Aelst L. The junctional multidomain protein AF-6 is a binding partner of the Rap1A GTPase and associates with the actin cytoskeletal regulator profilin. *Proc Natl Acad Sci USA*. 2000;97:9064–9069.
19. Stiemke MM, McCartney MD, Cantu-Crouch D, Edelhauser HF. Maturation of the corneal endothelial tight junction. *Invest Ophthalmol Vis Sci*. 1991;32:2757–2765.

20. Ottersen OP, Vegge T. Ultrastructure and distribution of intercellular junctions in corneal endothelium. *Acta Ophthalmol* (*Copenh*). 1977;55:69–78.
21. Petroll WM, Hsu JK, Bean J, Cavanagh HD, Jester JV. The spatial organization of apical junctional complex-associated proteins in feline and human corneal endothelium. *Curr Eye Res*. 1999;18:10–19.
22. Stern ME, Edelhauser HF, Pederson HJ, Staatz WD. Effects of ionophores X537a and A23187 and calcium-free medium on corneal endothelial morphology. *Invest Ophthalmol Vis Sci*. 1981;20:497–508.
23. Nartey IN, Cavanagh HD, Jester JV, Andrews P, Petroll WM. Characterization of specular "dark events" in human donor corneal endothelium by scanning and transmission electron microscopy. *Cornea*. 1998;17:544–549.
24. Hsu JK, Cavanagh HD, Jester JV, Ma L, Petroll WM. Changes in corneal endothelial apical junctional protein organization after corneal cold storage. *Cornea*. 1999;18:712–720.
25. Zen K, Babbin BA, Liu Y, Whelan JB, Nusrat A, Parkos CA. JAM-C is a component of desmosomes and a ligand for CD11b/CD18-mediated neutrophil transepithelial migration. *Mol Biol Cell*. 2004;15:3926–3937.
26. Bazzoni G, Martinez-Estrada OM, Orsenigo F, Cordenonsi M, Citi S, Dejana E. Interaction of junctional adhesion molecule with the tight junction components ZO-1, cingulin, and occludin. *J Biol Chem*. 2000;275:20520–20526.
27. Williams LA, Martin-Padura I, Dejana E, Hogg N, Simmons DL. Identification and characterisation of human junctional adhesion molecule (JAM). *Mol Immunol*. 1999;36:1175–1188.
28. Kornecki E, Walkowiak B, Naik UP, Ehrlich YH. Activation of human platelets by a stimulatory monoclonal antibody. *J Biol Chem*. 1990;265:10042–10048.
29. Aurrand-Lions M, Johnson-Leger C, Wong C, Du Pasquier L, Imhof BA. Heterogeneity of endothelial junctions is reflected by differential expression and specific subcellular localization of the three JAM family members. *Blood*. 2001;98:3699–3707.