HAVcR-1 Reduces the Integrity of Human Endothelial Tight Junctions

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Abstract. *Background: Hepatitis A virus cellular receptor-1 (HAVcR-1) is the cellular receptor for Hepatotropic picornavirus. Although HAVcR-1 is expressed in every human organ, its natural function remains unknown. This study investigated the location, association and functionality of HAVcR-1 in human endothelial cells. Materials and Methods: HAVcR-1 was either overexpressed or knocked-down by plasmid electroporation in human umbilical vein endothelial (HECV) cells. Changes in tight junction (TJ) behaviour were assessed using transendothelial resistance, and paracellular permeability. Binding partners and the location of HAVcR-1 protein was assessed using Western blotting/immuno-fluorescence. Results: HAVcR-1 co-localised with zonula occludens-1 (ZO-1) and ZO-2 proteins, both of which are involved in the formation, maintenance and function of TJ. The overexpression of HAVcR-1 resulted in reduced TJ formation; knockdown cells were resistant to hepatocyte growth factor (HGF)-mediated TJ disruption. HGF was unable to effect reduced resistance in these cells. HAVcR-1 was co-precipitated with the TJ regulatory factor Ras homolog gene family, member C (Rho C). Conclusion: HAVcR-1 may have a novel function as part of the regulatory apparatus for TJ in human endothelial cells.*

Hepatitis A virus cellular receptor (HAVcR-1) (1), also known as KIM-1 (kidney injury molecule-1) and TIM-1 (T-cell Ig- and mucin domain-containing molecule-1), is the cellular receptor for *Hepatotropic picornavirus*, which causes acute hepatitis A in humans. HAVcR-1 is a class I integral membrane glycoprotein with an extracellular domain containing an *N*-terminal cysteine-rich region, followed by a extended *O*-glycosylated threonine-, serine- and proline-rich region that makes it accessible for interactions with extracellular molecules. Human HAVcR-1 is expressed in every human organ including the liver, small intestine, colon and spleen, and is expressed at higher levels in the kidney and testis. Although the natural function of HAVcR -1 remains unknown, the TIM-1 gene, which is found on mouse chromosome 11 and is homologous to a segment of human chromosome 5q31-33, has been associated with disease susceptibility.

Virus receptors on membranes are often associated with junctional structures such as adherens and tight junctions (TJ), for example junctional adhesion molecule-1 (JAM-1), Coxsackie-adenovirus receptor (CAR) and members of the nectin family. JAM has been identified as a receptor for reovirus (2) and JAM-1 protein has been noted to be redistributed from TJs under the influence of cytokines on endothelium (3, 4). In addition, the CAR enables the attachment of the adenovirus *via* the interaction of the adenovirus finger-knob (5) and although its role is poorly understood it has been suggested that it is associated with the TJ structure in normal cells (5). Furthermore, human nectin is identical to the poliovirus receptor-related protein (6) and has been identified as an alphaherpes virus entry mediator (7). It is distributed in the cell–cell junction area (8) where afadin, an actin filament-binding protein, connects the nectin proteins to the actin cytoskeleton.

ZO-1, a member of the zonula occludens family, is an essential component of both TJ and adherens junctions. ZO-1, ZO-2 and ZO-3 are 220, 160 and 100 kDa, respectively. Both ZO-2 and -1 bind to ZO-3. ZO-1, -2 and -3 constitute the undercoat structure of the TJ, together with other peripheral proteins (9). ZO-2 and -3 interact with F-actin (10). TJ affiliations with the apical F-actin cytoskeleton are crucial in regulating TJ function. F-actin organization is influenced by the Ras homolog gene family (Rho) GTPase family, which also controls TJ function. GTPase is able to regulate TJ assembly and Rho inhibition results in decreased localisation of ZO-1 and occludin to the cell junction;

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constitutive Rho signalling causes an accumulation of ZO-1 and occludin at cell junctions (11).

This study examined the location, binding partners and of HAVCR-1. We also investigated the effect of HGF, a cytokine we have previously shown to modulate TJs in endothelial cells (12).

Materials and Methods

Cell lines. The HECV, human umbilical vein endothelial cell line, was obtained from ICLC (Interlab Cell Line Collection Genova, Italy) and routinely maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma Ltd, Poole, Dorset, UK) supplemented with 10 % foetal calf serum (FCS), penicillin and streptomycin (Sigma-Aldrich Ltd, Poole, Dorset, UK). Blasticidin (Melford, Laboratories Ltd., Ipswich, UK) was added for the maintenance of transfected cells.

Materials. HAVcR-1 monoclonal antibodies were obtained from R & D systems (Abingdon, Oxford, UK). Polyclonal antibodies to occludin, ZO-1, ZO-2 and RhoC were obtained from Insight Biotechnology Ltd (Wembley, Middlesex, UK). Human recombinant hepatocyte growth factor (HGF) was a gift from Dr T Nakamura, Osaka, Japan. HGF is a potent mitogen, motogen and angiogenic factor which has been well described in disrupting TJ in endothelial cells (13). All other materials were from Sigma Ltd unless otherwise stated.

RT-PCR. RNA extraction was performed using an RNA extraction kit (Advanced Biotechnologies Ltd, Nottingham, UK). RT-PCR was performed using a reverse transcription kit (Advanced Biotechnologies Ltd) according to the manufacturer's instruction. Cells were screened for the expression of the HAVcR-1 gene by PCR using the following primers (synthesized by Invitrogen, Paisley, N. Ireland). HAVcR-1 forward primer (5'-3') ATGCATCCTCAAGTGGTC; HAVcR-1 reverse primer (5'-3') ACCCCAATCTTCATGTCCACATT. Amplification for HAVcR-1 was carried out by: denaturing at 94˚C for 5 min; 36 cycles at 94˚C for 40 s; 54˚C for 40 s; 72˚C for 60 s and a final extension period of 72˚C for 10 min. Plasmids incorporating the hammerhead ribozyme transgene specifically targeting human HAVcR-1 were constructed and used to knockdown the HAVcR-1 transcript. Ribozymes to HAVcR-1 were created using the following primers: HAVcR1rib2R ACTAGTGGAGAGGAGGTCCATCTG TTTCGTC CTCACGGACT and HAVcR1rib2F CTGCAGTAG TGGCAGG GTAGTGTCTGATGAGTCCGTGAGGA.

TOPO TA cloning and electroporation. Cloning was performed using a pEF6/V5-His TOPO TA Cloning® cloning kit (Invitrogen) in accordance with the manufacturer's instructions. Overexpression plasmids and ribozyme plasmids were electroporated into wild-type HECV cells using electroporation (Equibio Easyject Plus; Equobio, Ashford, Middlesex, UK). An expression control plasmid ($HECV^{plas}$) was also electroporated into wild-type HECV cancer cells as a vector-only control.

Western blotting analysis. Overexpression and knockdown of HAVcR-1 was confirmed by Western blotting. The proteins extracted were quantified using fluorescamine (Sigma, Poole UK) and a fluorescent plate reader (Denley Instruments, Colchester, Essex, UK). Equal amounts of protein were resolved on an 8% SDS-PAGE and electroblotted onto nitrocellulose membrane (Hybond; Amersham, Hertfordshire, UK) and probed with anti-HAVcR-1 antibodies. Primary antibodies were detected by peroxidase-conjugated immunoglobulins (1:1000) and an enhanced chemiluminescence system (Insight Biotechnologies). For immunoprecititation, cells were lysed after treatment, with cells treated with orthovanadate used as a positive control. Cell debris was removed by centrifugation (13,000 g for 10 min) and anti-ZO-1, anti-ZO-2, anti-ZO-3, anti-occludin and anti-RhoC was added to each sample of equal protein concentration. Protein A/G-agarose was added after 1 h and incubated at 4˚C overnight. The precipitates were washed three times and lysed in sample buffer. After Western blotting, the membrane was probed with anti-HAVcR-1 antibodies.

Immunofluorescent staining of HECV cells. The HECV cells were grown in 16-well chamber slides (Lab-Tek, Fisher Scientific UK Ltd, Loughbourgh, Leicestershire, UK). Briefly, 300-400,000 cells/ml in complete medium were added to duplicate wells (30,000 cells/well) in the presence or absence of HGF (40 ng/ml). After incubation (0-24 h) the cells were fixed with 4% paraformaldehyde, washed and permeabilised with 0.1% Triton X-100. The wells were rinsed again and blocked in 10% blocking buffer and incubated with the appropriate primary antibodies for 1 h and secondary antibodies for 50 min. TRITC or FITC fluorescently stained cells were mounted with FluorSave (Calbiochem-Novabiochem Ltd, Nottingham, UK) reagent and visualised using a scanning laser confocal microscope (Leica Microsystems, Heidelberg GmbH, Germany) under a 40×1.0 oil immersion lens objective. Representative images were taken of the endothelial cells.

Transendothelial resistance (TER). TER was measured with an EVOM voltohmmeter (EVOL, World Precision Instruments, Aston, Hertfordshire, UK), equipped with a pair of STX-2 chopstick electrodes (WPI, Sarasota, FL, USA) as previously described (13). Inserts without cells, inserts with cells in medium, and inserts with cells with or without HGF at 40 ng/ml, were tested for a period of 2 h (13).

Paracellular cell permeability (PCP). This was determined using fluorescence-labelled dextran, FITC-Dextran 40 (Sigma-Aldrich Ltd), molecular weight 40 kDa (13). HECV cells were prepared and treated as in the TER study, but with the addition of Dextran-40 to the upper chamber. Media from the lower chamber was collected at intervals up to 2 h and fluorescence from these collections was read on a multichannel fluorescence reader (Denley).

Statistical analysis. Significance was determined using Student's *t*-test and ANOVA analysis using Microsoft Excel (Office XP). $P<0.05$ was accepted as statistically significant difference.

Results

Expression and knockdown of HAVcR-1. The HAVcR-1 gene was successfully amplified from normal breast tissue (Figure 1, left panel) and cloned for subsequent investigation. A ribozyme transgene was also successfully created. Following transformation of the human endothelial cell line HECV with the HAVcR-1 gene or the ribozyme, overexpression ($HECV^{HEX}$) or knockdown ($HECV^{HKO}$) of the gene was demonstrated by the presence or absence of mRNA (by RT-PCR) (Figure 1, right panel).

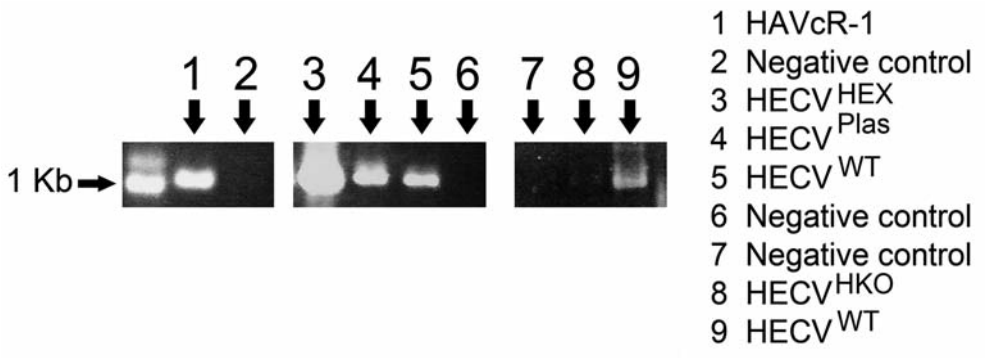

Figure 1. *Amplification of the HAVcR-1 gene for cloning (left panel). Successful cloning and overexpression of HAVcR-1 in HECV cells (middle panel). Knockdown of HAVcR-1 confirmation in HECV cells (right panel). HEX: HAVcR-1 overexpression plasmid, WT: wild type, HKO: HAVcR-1 knockdown.*

HAVcR-1 immunoprecipitation. Immunoprecipation experiments revealed that the ZO-1 *C*-terminus (lane 8) the and ZO-2 *C*-terminus (lane 7) associated with the 55 kDa HAVcR-1 protein, as shown in Figure 2A. In addition, immunoprecipitation of the HAVcR-1 protein from wild-type HECV cells revealed that HAVcR-1 was also associated with occludin at its *N*-terminus (Figure 2, Lane 3) and that the 30 kDa Rho C protein also immunoprecipitated with HAVcR-1 (Figure 2A, left panel).

Effect of HAVcR-1 knockdown. Dual-immunfluorescence staining of ZO-1^{TRITC} and HAVcR-1^{FITC} in HECV wild-type and transfected cells showed strong expression of ZO-1 between the cells and a cytoplasmic distribution of HAVcR-1 (Figure 2A). In the untreated cells, ZO-1 was evenly distributed between all the cells, including the HAVcR-1 knockdowns ($HECV^{HKO}$) (Figure 2B, see arrows). Disruption of the TJs in these cells was mediated by the addition of HGF in order to observe any change on localisation of HAVcR-1. After treatment with HGF, the wild-type cells showed a loss of expression and disrupted ZO-1 distribution between the cell–cell junctions (Figure 2C). In contrast, $HECV^{HKO}$ showed a stronger and more concentrated ZO-1 expression pattern, as indicated by the arrows (Figure 2BD).

HAVcR-1 and TJ function TER. TER was measured over a period of 2 h to determine what effect, if any, overexpression ($HECV^{HEX}$) and knockdown of HAVcR-1 ($HECV^{HKO}$) had on human endothelial cell TJ function.

Wild-type endothelial cells ($HECV^{WT}$) showed some increase in resistance over the 2 h period, as is usually observed (change in TER (Ω) for $HECV^{WT}$: 12.66±3.79 after 15 min; 13.67±3.06 after 30 min; 14.33±2.53 after 60 min; 18±2 after 120 min) (Figure 3A). Both transformed cells types showed little change in TER over the same time (change in TER (Ω) for $HECV^{HEX}$: –1.67±2.65 after 15 min; 0.33±2 after 30 min; -0.67±6.08 after 60 min; 1.33±1.73 after 120 min: change for $HECV^{HKO}$: –0.67±4.04 after 15 min; –3.33±2 after 30 min; 1.67±7.81 after 60 min; 3±2.08 after 120 min).

There was, however, a significant difference in TER of the $HECV^{HKO}$ cells in response to treatment with the pro-angiogenic factor HGF compared to both the $HECV^{WT}$ and $HECV^{HEX}$ cells (Figure 3B). As expected, the $HECV^{WT}$ cells exhibited a reduction in TER on treatment with HGF (40 ng/ml) as the TJ structure was broken down (HGF-treated $HECV^{WT}$: –39±2.52 after 15 min; –59±1.53 after 30 min; –88±4.16 after 60 min; –90±5.51 after 120 min). The $HECV^{HEX}$ cells had a slightly more reduced TER after 120 min, compared to the wild-type HECV cells in response to HGF (HGF-treated $HECV^{HEX}$: –51±7.09 after 15 min; –63±4.9 after 30 min; –77±3.6 after 60 min; –107±2.08 after 120 min). HGF produced significantly less response in the $HECV^{HKO}$ cells (HGF-treated $HECV^{HKO}$: 4.33±3.04 after 15 min; 7±2 after 30 min; 5.67±3.21 after 60 min; –1.33±2.1 after 120 min, $p<0.01$).

HAVcR-1 and TJ function, PCP was measured over a period of 2 h to determine what effect, if any, overexpression ($HECV^{HEX}$) and knockdown of HAVcR-1 ($HECV^{HKO}$) had on human endothelial cell paracellular pathway function.

Wild-type endothelial cells ($HECV^{WT}$) showed some decrease in PCP over the 2 h period, as is usually observed (change in TER (relative fluorescence units, RFU) for $HECV^{WT}$: –290±169 after 60 min; –190±206 after 120 min) (Figure 3C). Both transformed cell also showed similar changes in PCP over the same time (change in PCP (RFU) for $HECV^{HEX}$: –162±79 after 60 min; –219±144 after 120 min: change for $HECV^{HKO}$: –289±225 after 60 min; –277±48 after 120 min).

Unlike the changes in TER measurement, there was no significant difference in PCP of the $HECV^{HKO}$ cells in response to treatment with the pro-angiogenic HGF compared to the

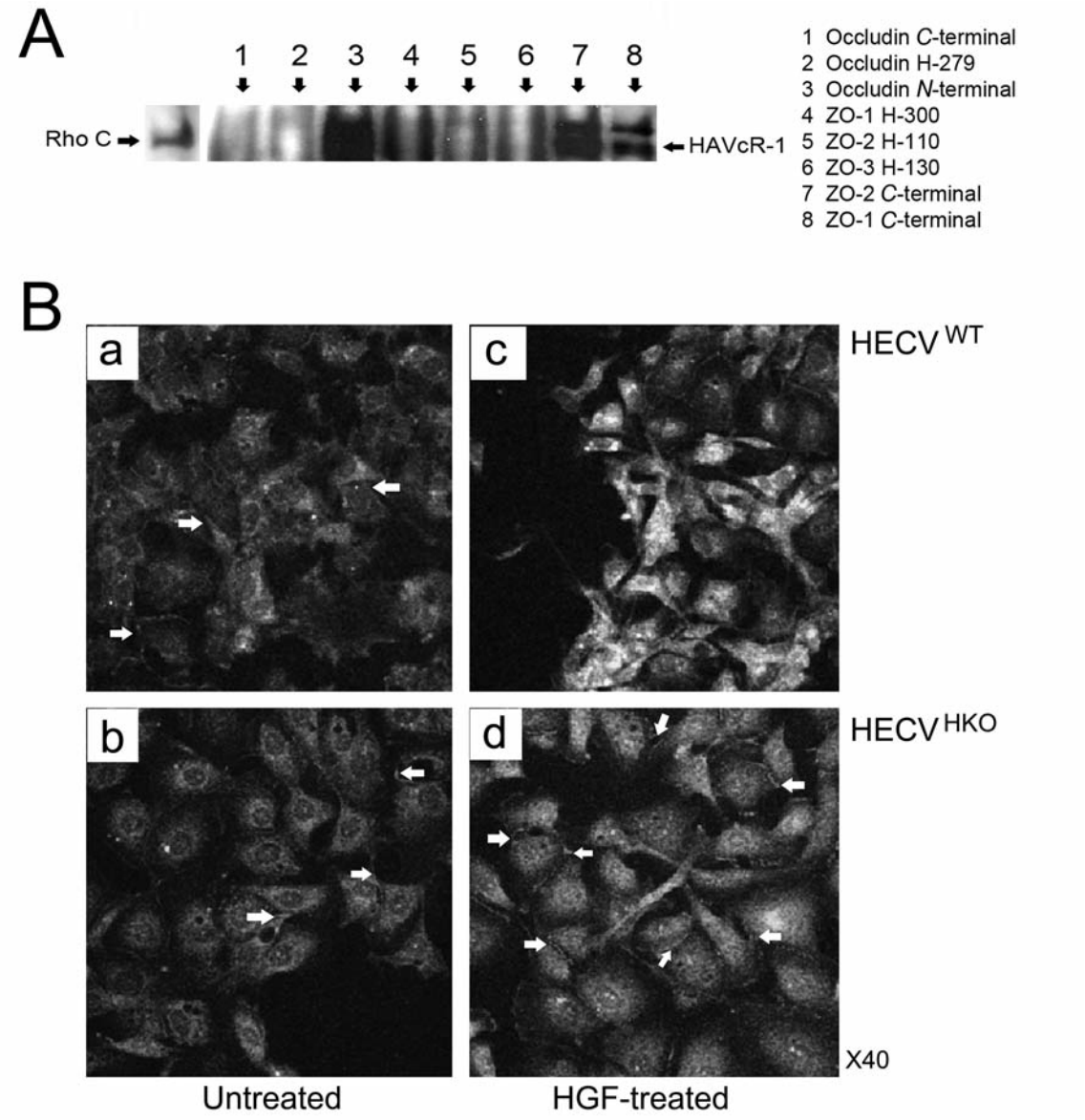

Figure 2. *(A) Western blots of HAVcR-1 immunoprecipitations. HAVcR-1 precipitated Rho C (left panel), ZO-2, and ZO-1 (right panel). (B) Confocal microscopy of HECV cells dual-stained with HAVcR-1 (green) and ZO-1 (red). Untreated WT cells show co-localisation (a) and HKO (HAVcR-1 knockdown) show the same close cell–cell association (b). Hepatocyte growth factor (HGF)-treated cells show markedly different distributions of HAVcR-1 and ZO-1 (c and d).*

$HECV^{WT}$ and $HECV^{HEX}$ cells (Figure 3D). As expected, the $HECV^{WT}$ cells exhibited a reduction in PCP on treatment with HGF (40 ng/ml) (HGF-treated $HECV^{WT}$: –565±142 after 60 min; –637±97 after 120 min). Neither the $HECV^{HEX}$ nor the $HECV^{HKO}$ cells showed significant changes in PCP compared to each other, or when compared to the wild-type HECV cells, in response to HGF (HGF-treated $HECV^{HEX}$: –314±56 after 60 min; –209±86 after 120 min: HGF-treated $HECV^{HKO}$: –290±302 after 60 min; –426±167 after 120 min, $p>0.05$).

Discussion

In this study, one of the first to investigate HAVcR-1 location and function, HAVcR-1 was found to co-localise and co-precipitate with ZO-1 (strongly) and ZO-2 (weakly), both at the *C*-terminii. This suggested that HAVcR-1 is located to and forms part of the TJ complex in human endothelial cells.

TJs are characteristic structures of one-layered epithelia and endothelia and are of central biological importance as an

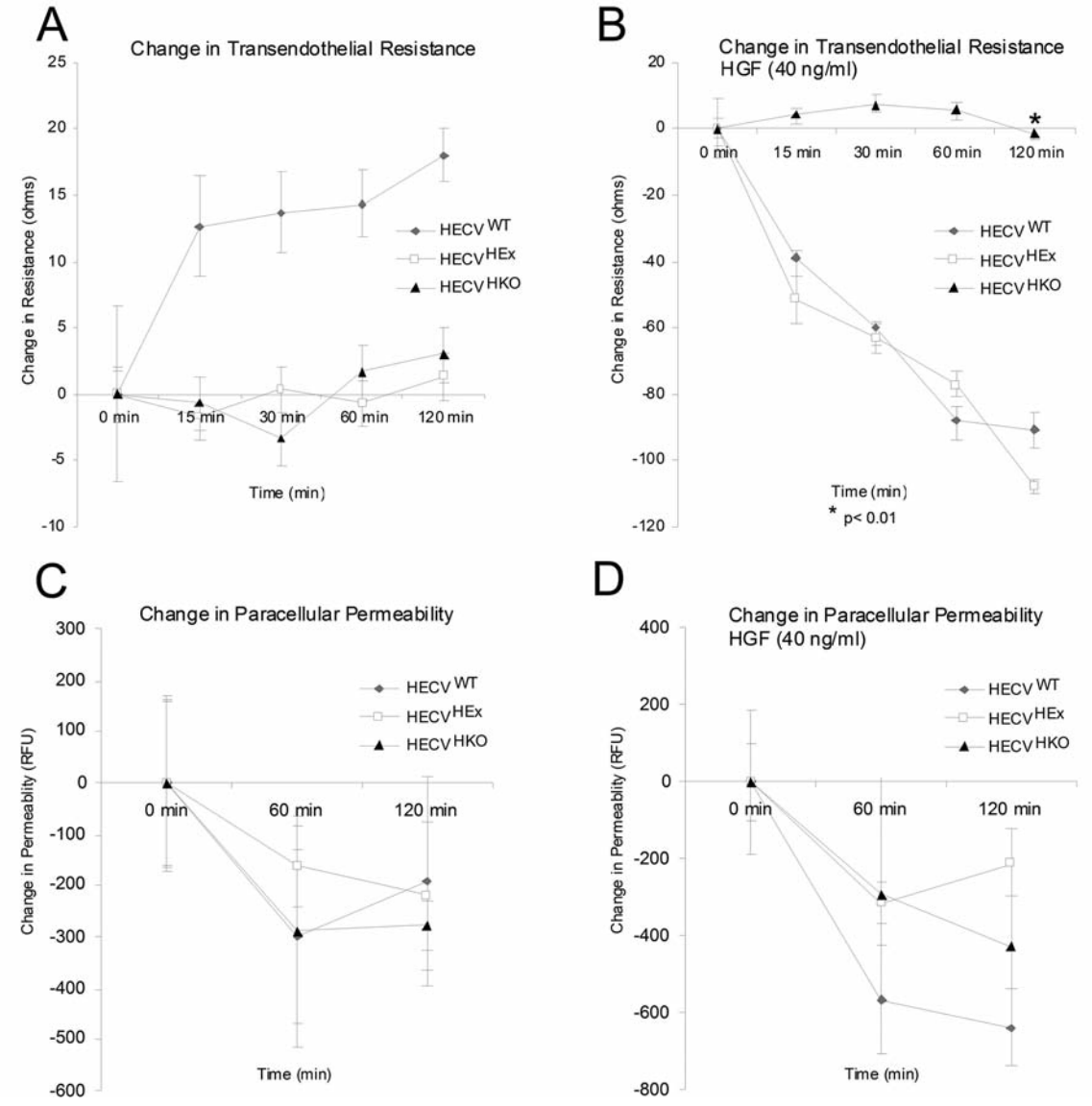

Figure 3. *Transendothelial resistance measurements of HECV cells untreated (A) and treated (B) with 40 ng/ml hepatocyte growth factor (HGF). Paracellular permeability of untreated (C) and HGF-treated (D) HECV cells. RFU: Relative fluorescence units.*

intramembranous 'fence' and as hydrophobic 'barriers' between lumina represented by liquid- or gas-filled spaces on one hand, and the mesenchymal space on the other (14). The TJ is the most apical component of the junctional complex of epithelial and endothelial cells (15). Cell adhesion to adjacent cells and the extracellular matrix is key not only to the organisation of epithelium into a tissue but also to the regulation of cellular processes such as gene expression, differentiation, motility and growth (16). Cell adhesion molecules, transmembrane receptors and the cytoskeleton, all of which are all organised into multimolecular complexes and the activation of signalling pathways, mediate their regulatory functions. Endothelial cells form a continuous monolayer *in vivo* which functions as a selective barrier to the passage of plasma proteins and extravasation of inflammatory cells (16). TJs exclusively carry out the structural organisation responsible for the paracellular permeability barrier function (16).

TJs are involved in two principal types of signal transduction process: signals are transduced from the cell interior towards forming or existing TJs to guide their assembly and to regulate their function; and signals are transmitted from the TJs to the cell interior to modulate gene expression, as well as cell proliferation and differentiation

(17). The molecular mechanisms that mediate these signal-transduction processes have only recently started to become unravelled; consequently, our knowledge of them is incomplete and entire pathways have yet to be completed (17). Often a component involved in signalling to the TJ is also involved in signalling from it. From the present study, it appears that HAVcR-1 may function as part of the transduction process, whereby the TJ structure is assembled/disassembled. Overexpression of the HAVcR-1 protein resulted in endothelial cells with reduced TJ integrity and an increased response to the HGF cytokine. Conversely, the knockdown of HAVcR-1 expression caused increased TJ assembly and a reduced response to HGF. This was observed from immunostaining of HAVcR-1, ZO-1 and ZO-2 in the cells and from changes in the TJ function assays. It should be noted that recent work has shown that occludin is required for a post-binding step in hepatitis C virus entry and infection (18), raising tantalising possibilities as to how HAVcR-1 might interact with occludin for HAVc entry into cells.

Interestingly, HAVcR-1 was also co-precipitated with RhoC, one of the Rho GTPase family. The family of Rho GTPases is a large subfamily of the Ras-superfamily, which have roles in numerous and diverse cellular processes, including polarisation, cell–cell and cell–matrix adhesion, membrane-trafficking, cytoskeletal and transcriptional regulation and cell proliferation (19). RhoC was originally identified as a promoter of metastatic behaviour (20-21), which was confirmed in later work (22-24). Rho GTPases are thought to regulate TJ assembly. Rho inhibition results in decreased localisation of ZO-1 and occludin at cell junctions; constitutive Rho signalling causes an accumulation of ZO-1 and occludin at cell junctions (11).

Overactivation of Rho, Rac and cell division control protein 42 homolog (Cdc42) with cytotoxic necrotizing factor 1 (CNF-1) seems to mirror key barrier-function disruptions previously reported for inactivation of RhoA (25). Rho GTPases have been implicated in the signalling by many vasoactive substances including thrombin, TNF-alpha, bradykinin, histamine, lysophosphatidic acid (LPA), vascular endothlelial growth factor (VEGF) and HGF. Rho and Rac have emerged as key regulators acting antagonistically to regulate endothelial cell barrier function: Rho increases actomyosin contractility, which facilitates the breakdown of intercellular junctions, whereas Rac stabilizes endothelial junctions and counteracts the effects of Rho (26). It has been suggested that RhoA activation induces an increase in TER which is mediated by Rho-associated kinase and at least one as yet unelucidated effector (27).

HAVcR-1 appears to have a role in the assembly and disassembly of endothelial TJs that may be mediated *via* the Rho GTPase signalling transduction apparatus. These instructions are then effected by changes in distribution of the ZO proteins (-1 and -2), thus causing changes in TJ structure and function in human endothelial cells. We propose this as a novel function for this previously described virus receptor. This additional and pertinent function is not without precedent however. It has been proposed that during evolution, viruses have taken advantage of a number of molecules that are involved in the adaptive immune system and that are all found as membrane-spanning proteins which have modern homologues in JAMs, Cobra cardiotoxin (CTX) and the nectin family (28). Generally, most of these were first described as virus receptors, with it becoming evident that they also have numerous other functions. This may also be the case for HAVcR-1. Further investigation into the mechanism for control and the cytoplasmic interactions of HAVcR-1 is ongoing, and may provide a key insight into the regulation of TJs in endothelial cells.

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