Interleukin-24 (IL-24) Expression and Biological Impact on HECV Endothelial Cells

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Abstract. *Background: IL-24, also termed MDA-7, is a member of the IL-10 family of cytokines. IL-24 is reported to be expressed in a series of cell lines, including keratinocytes as well as breast, lung and prostate cancer cells, but was primarily found in a human melanoma cell line. IL-24 is suggested to have many biological properties displaying anti-tumour effects via induction of apoptosis, suppressing proliferation, invasion and metastasis of cancer cells. IL-24 has also been reported to inhibit the migration of cancer cells and keratinocytes, and have anti-angiogeneic properties. The biological functions of IL-24 are regulated through both autocrine and paracrine methods. However, currently there exists little knowledge regarding the effect of IL-24 on endothelial cell biology. Materials and Methods: The impact of rhIL-24 on human endothelial HECV cell growth, migration, trans-endothelial resistance and angiogenic potential was examined using cellular functional assays. Additionally, the relationship between IL-24 and a number of cell junction proteins were examined using immunofluorescence staining. Results: IL-24 and receptor molecules was found to be expressed in HECV endothelial cells. Treatment of this cell line with rhIL-24 was found to promote cell migration rates and suppress tubule formation. Conclusion: Treatment of HECV cells with rhIL-24 can promote migration and inhibit tubule formation but does not impact cell growth or permeability at the tested concentrations. Potential links between IL-24 and AKT or PLCγ-related pathways with regard to these effects are also presented in the present study.*

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Key Words: IL-24, MDA-7, endothelial cell, HECV.

Interleukin (IL) 24, also known as melanoma differentiation-associated 7 (MDA-7), was initially found in human melanoma cells and is a member of the IL-10 family of cytokines (1). The *MDA-7* gene is located to a 195-kb area and codes for IL-24. Secreted mature IL-24 is a glycoprotein and has a predicted size of 35-40 kDa (2). Studies show that IL-24 is expressed in the human immune system, including the thymus, spleen, normal melanocytes and peripheral blood leukocytes (3, 4, 2). In a recent report, IL-24 was found expressed in keratinocytes during the early phases (day 2 and day 6 after wounding) but no expression was observed in the latter stages of wound repair (5).

In early studies IL-24 was recognized as an associated factor of differentiation, growth, and apoptosis and was also seen to have anti-tumour effects (6). In a breast cancer cohort, lower levels of IL-24 were associated with a poorer patient prognosis and treatment of breast cancer cells with rhIL-24 resulted in decreased breast cancer cell motility *in vitro* (7). It has been reported that IL-24 inhibited human lung tumour growth through reduction of tumour vascularisation in a mouse xenograft model (8). A recent study also suggested that IL-24 can reduce *in vivo* tumour growth of prostate cancer and decrease angiogenesis in mouse xenograft models, observations that were associated with enhanced expression of nuclear clusterin (nCLU) and a reduction in the expression of soluble clusterin (sCLU) (9). A number of studies have suggested that IL-24 may enhance apoptosis in certain cancer cell lines (10-12) and IL-24 has been shown to suppress growth and metastasis of tumours through interference with tumour vasculature and inhibition of signal(s) produced by tumour cells (13).

In addition to its anti-tumour properties, IL-24 has other biological functions and is regulated in both autocrine and paracrine manners. IL-24 expression has been demonstrated in human peripheral blood monocytes (PBMC), T-cells and macrophages whilst expression of IL-24 in PBMC following stimulation with antigens or mitogens is due to cytokine

Table I. *Primers used in the study.*

Primer	Forward	Reverse
IL-24	TTCTCTGGAGCCAGGTATC	TAGAATTTCTGCATCCAGGT
IL-20R1	TCCAGAGAAGTGGAAGAGAA	ATCACTGCTTTTTCCCAGTA
IL-20R2	CAACATGAAGCATCTCTTGA	CACCTCCACACATTCTGTCT
IL-22R1	TCGATTCAGATGATTGTTCA	CTGATCTGGGAGTACTGGAC
GAPDH	AGCTTGTCATCAATGGAAAT	CTTCACCACCTTCTTGATGT

stimulation, suggesting that IL-24 is related to a cytokine network expressed in the stimulation response to the human cellular immune system (14).

Ectopic expression of IL-24 can inhibit the differentiation of endothelial cells (8) and IL-24 has also been reported to have a significant inhibitory effect on keratinocyte migration, slowing wound healing through an AKT-dependent pathway (15).

It was reported that IL-24 could stimulate human $CD8^+$ T-cells to induce the product of interferon (IFN)-γ mainly through interaction with the IL-24 receptors present on $CD8^+$ T-cells (16). The cytokine and anti-tumour activity of IL-24 is similar to other Th1-type cytokines, such as IFN-γ and IL-12. It was indicated that IL-24 acted as a pro-Th1 cytokine and led to production of tumour necrosis factor α, IL-6 and IFN-γ (17-19). Studies have shown that IL-24 can be secreted *via* stimulated peripheral blood monocyte cells and regulates its function *via* two heterodimic receptors:IL-20R1/IL-20R2 and IL-22R1/IL-20R2 (14, 20).

In the present study, we evaluated the biological implications of IL-24 on HECV cells and identified the expression of its receptors in HECV cells. We conclude that IL-24 significantly increases the migration of HECV cells after wounding and has an inhibitory effect on HECV cell tubule formation, whilst no obvious impact on cell barrier function or growth rates at tested concentrations. Our data also implicate potential interaction of IL-24 with PLCγ and AKT signaling pathways.

Materials and Methods

Materials and cell lines. Human HECV cells were purchased from Interlab (Milan, Italy). Cells were routinely cultured in Dulbecco's modified eagle medium (DMEM) containing 10% FCS, penicillin, streptomycin and amphotericin B at 37˚C in 5% CO_2. Recombinant human interleukin IL-24 (rhIL-24) was obtained from R&D Systems Europe (Abingdon, UK). AKT inhibitor was obtained from Calbiochem (Nottingham, UK). PLCγ inhibitor was obtained from Calbiochem.

Reverse transcription and polymerase chain reaction. RNA was extracted from HECV cells using TRI reagent in accordance with the manufacturers guidelines (Sigma, Dorset, UK). Following extraction RNA was re-suspended in DEPC water and quantified using a spectrophotometer (WPA UV 1101, Biotech, Photometer, Cambridge, UK). Reverse transcription was undertaken to generate a cDNA copy from the RNA template using a high capacity cDNA reverse transcription kit (Life Technologies, Paisley, UK) in accordance with the manufacturers guidelines. Polymerase chain reaction was undertaken using Go Taq Green 2X mastermix (Promega, Southampton, UK), specific primer pairs (Table I), PCR water and cDNA. The reaction was carried-out in an Applied Biosystems 2720 thermocycler (Life Technologies, Paisley, UK). PCR conditions were as follows; initial denaturation 94˚C, 5 min; 36 cycles of 94˚C for 30 sec, 55˚C for 40 sec and 72˚C for 60 sec; final extension of 72˚C for 10 min. Subsequently, PCR products were loaded onto an agarose gel (1%) and electrophoretically separated before being stained in Sybr Safe DNA gel stain (Life Technologies, Paisley, UK) for 30 min and visualed using a U:Genius gel doc system (Syngene, Cambridge, UK).

Electric cell-substrate impedance sensing (ECIS)-based migration assay. An ECIS system was used to quantify cell migration (ECIS ztheta, system; Applied Biophysics Inc., NJ, USA). This technique has been previously described (15, 21). In brief, ECIS arrays were seeded with identical numbers of HECV cells (10^5cells/well). Groups of wells were treated with a combination of 50 ng/ml, rhIL-24, AKT inhibitor (200 μM), PLCγ inhibitor (10 μM) or left untreated as a control. The array was then attached to the ECIS system and resistance recorded as the cells attached and spread. Following formation of a monolayer, the cells were electrically wounded through the application of 2,000uA for 20 secs per well, resulting in a defined "wound" in the monolayer. The change in resistance as cells migrated to close the wound was recorded over a minimum of 4 hours and was used as a measure of cellular migration.

Measurements of transendothelial electrical resistance (TER). The impact of IL-24 on cellular barrier property was performed using TER across confluent cell monolayers, as modified from a previously described method (22). Briefly, 400,000 HECV cells were seeded in 24-well plate inserts (containing 0.4-μm pores) and medium was added in and below the inserts before incubating the plates overnight to obtain a confluent monolayer. Cells were treated with 50 ng/ml IL-24 or untreated. The TER across the monolayer was recorded using a EVOM2 volt-ohmmeter (World Precision Instruments, Hitchin, UK) at 30-min intervals over 210 min.

Tubule formation assay on Matrigel. A tubule formation assay was used to assess the ability of IL-24 to impact the tubule formation of HECV cells. This method has been previously reported (22). In brief, 100 μl Matrigel (1:10 dilution) was added to each well of a

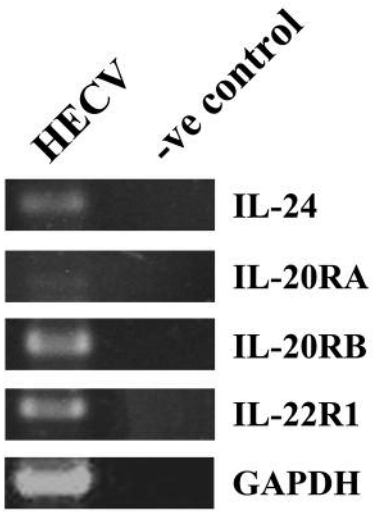

Figure 1. *RT-PCR screening of IL-24 and its receptors in HECV cells. IL-24 together with IL-20RB and IL-22R1 receptor expression was observed in the HECV cell line. IL-20RA expression was only weakly detectable.*

96-well plate and incubated overnight to dry the Matrigel. Subsequently, HECV cells were seeded in 100 µl of medium onto this layer and returned to the incubator for 1 h before an additional layer of Matrigel (100 µl; 1:10 dilution) was added and further incubated for 30 min. Following incubation, 100 µl of either rhIL-24 (50 ng/ml) or normal medium was added and the assay incubated for 24 h. After this period, the medium was aspirated and fixed with 4% formalin before images were captured under the microscope in at least three random fields. Internal perimeters of tubules were calculated using the image J software.

In vitro cell growth assay. HECV cells were seeded (3,000 cells/well) into a 96 well plate and treated either with 50 ng/ml rhIL-24 or left untreated in normal medium. Replicate plates were prepared as either a reference or three-day treatment incubation. Following appropriate incubation, medium was removed, the cells were fixed in 4% formalin and stained in 0.5% (w/v) crystal violet. The crystal violet stain taken-up by the cells was extracted in 10% acetic acid and the absorbance of this solution measured in a spectrophotometer plate reader (Bio-Tek Instruments Inc, Vermont, USA). Absorbance values were taken as indicative of cell growth and percentage increases calculated based on the reference plate.

Immunofluorescence assay. HECV cells were seeded in a 16-well chamber slide (32,000 cells/well) and incubated overnight at 37˚C. Subsequently, the medium was aspirated and replaced with either normal medium (control) or with medium containing 50 ng/ml IL-24 and incubated for 1 h before cells were fixed in 4% formalin. The cells were then rehydrated in BSS for 20 min at room temperature, permeabilised in 0.1% Triton X100 for 3 min and blocked in blocking buffer (TBS containing horse serum) for 30 min. Subsequently, cells were washed in wash buffer twice before primary antibodies (ZO-1, ZO-2, Occludin, Claudin-1 or Claudin-5; Insight Biotechnology Ltd., Middlesex, UK) were added and incubated for 1 h. The primary antibody was then washed-off the cells through three washes with TBS before TRITC fluorescently conjugated secondary antibodies, specific to the primary antibody species (Sigma, Dorset, UK) were added and incubated for 30 min in the dark. Finally, cells were washed three times in TBS and mounted in Fluorosave mounting medium (Calbiochem-Novabiochem Ltd., Nottingham, UK), left overnight and visualised under a fluorescent microscope (Olympus BX51, Southend-on-sea, UK).

Statistical analysis. Sigma Plot 11 statistical software package (Systat Software Inc., London, UK) was used to analyze experimental data and identify statistical differences between groups. Experimental procedures were repeated independently and comparisons drawn between the IL-24 treatment groups with untreated controls using appropriate statistical tests based on the assay and normality of data. A value of $p<0.05$ was deemed statistically significant.

Results

Expression of IL-24 and receptors in HECV cells. IL-24 together with the IL-20RA, IL-20RB and IL-22R1 receptor expression was examined in HECV (Figure 1). IL-24 was found to be expressed in the HECV cell line. Additionally, IL-20RB and IL-22R1 receptors were also expressed, though only weak expression was noted for IL-20RA.

IL-24 treatment has little impact on transendothelial resistance (TER) and growth of HECV cells. Cell growth rates following treatment with rhIL-24 were examined (Figure 2A). Compared to the untreated control group, treatment of HECV cells with rhIL-24 (50 ng/ml) had no significant effects on cell growth rates over a 3 day incubation ($p>0.05$) suggesting that, at this concentration, IL-24 does not impact the growth of HECV cells. The potential of IL-24 to alter the barrier function of HECV cells was also examined through detection of transendothelial resistance (TER) (Figure 2B). Results indicated that treatment with rhIL-24 (50 ng/ml) had little effect on the TER of HECV cells with little observable differences being seen in comparison to the untreated controls and no significant differences observed.

IL-24 enhances HECV cell migrations rates. The ECIS system was used to detect HECV cell migration rates following electrical wounding of the cell monolayer in response to rhIL-24 (50 ng/ml) (Figure 2C). HECV cell migration rates were enhanced following treatment with rhIL-24 compared to the untreated control ($p<0.001$). In order to explore possible pathways linked to the pro-migratory effect of IL-24 on HECV migration, we used two small molecule pathway inhibitors [AKT inhibitor (200 µM) and PLCγ (10 µM)] as a trial target to explore downstream effectors of IL-24 (Figure 2C). Inhibitors were added in

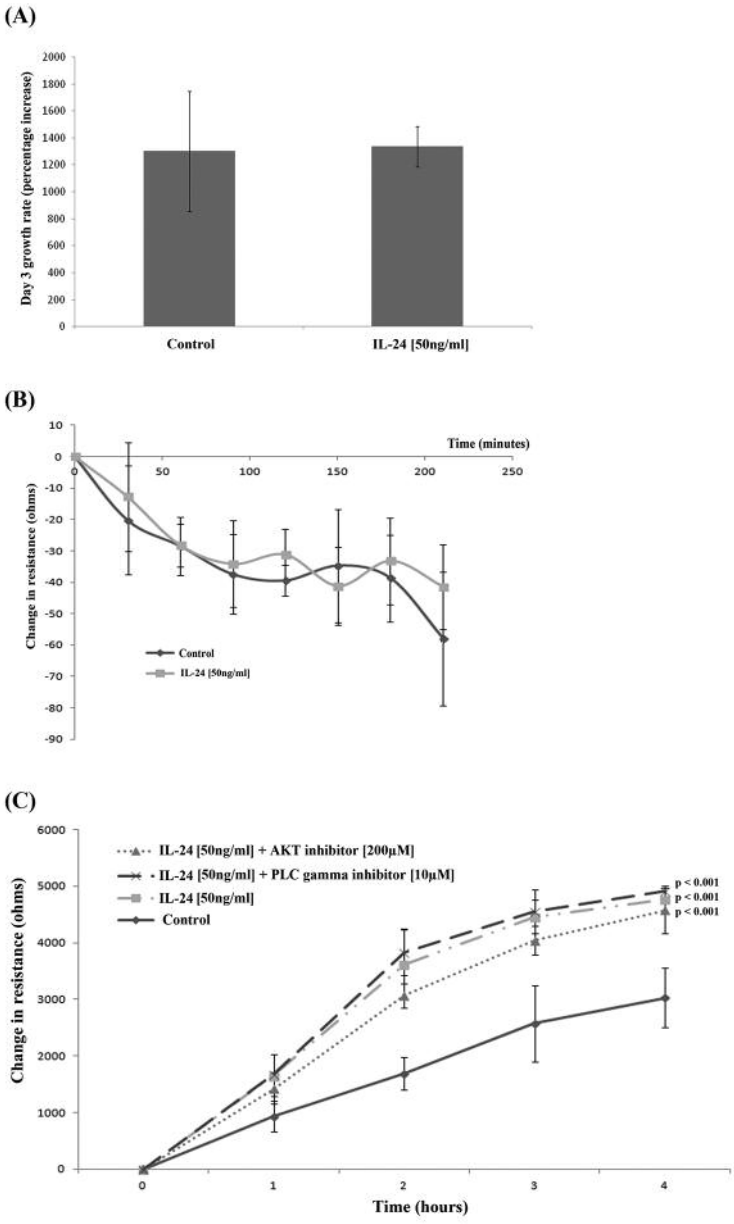

Figure 2. *Effects of rhIL-24 on the cellular function of HECV cells. (A) Treatment of HECV cells with 50 ng/ml rhIL-24 did not impact on HECV cell growth in a 3-day in vitro growth assay. (B) Transendothelial resistance (TER) of a HECV cell monolayer was similarly unaffected by treatment with 50 ng/ml rhIL-24. (C) Migration rates following electrical wounding of the HECV monolayer were significantly increased, in comparison to untreated control cells, following treatment with 50 ng/ml rhIL-24. Co-treatment of HECV cells with 50 ng/ml rhIL-24 and either the PLCγ inhibitor or the AKT inhibitor similarly enhanced cell migration in comparison to untreated control cells. A slight reduction in migration rates was seen between rhIL-24-treated cells and those co-treated with rhIL-24 and AKT inhibitor. Mean values±SEM show statistical significance in comparison to untreated control cells is indicated.*

addition to the 50 ng/ml rhIL-24 treatment. Upon combination of rhIL-24 treatment with the AKT or PLCγ small-molecule inhibitors the pro-migratory effect of rhIL-24 remained, with all IL-24/inhibitor combination groups showing significantly higher rates of migration in comparison to the untreated controls ($p<0.001$). In comparison to the individually rhIL-24-treated group, no further significant alterations in migration were seen in either of the rhIL-24/inhibitor co-treated groups. However, it was noted that, whilst not significant, the addition of AKT inhibitor in combination to IL-24 did generally reduce the pro-migratory impact of IL-24 alone.

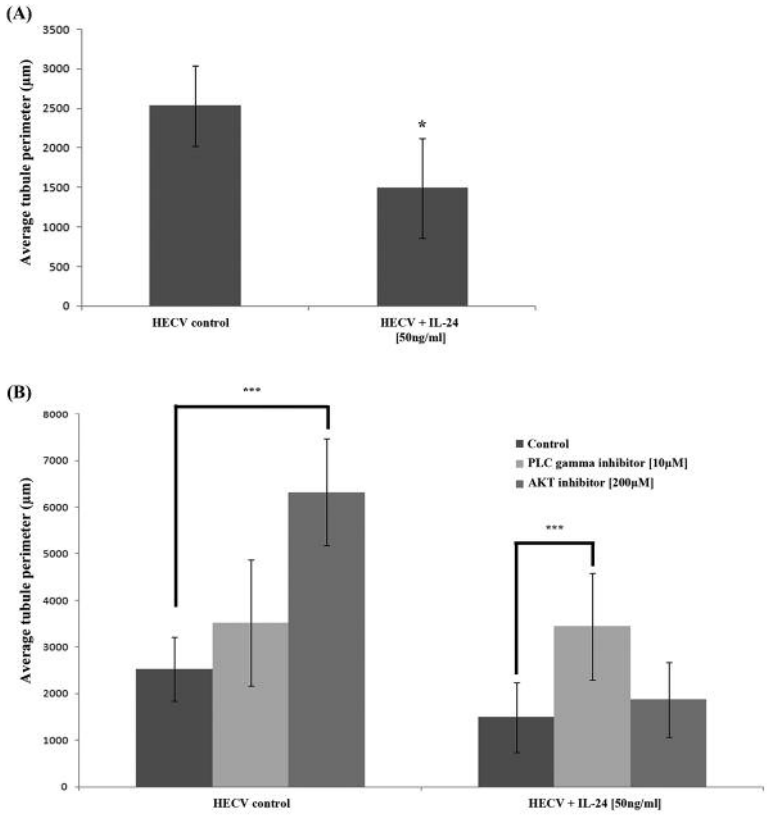

Figure 3. *Impact of rhIL-24 on HECV tubule formation. (A) Treatment of HECV cells with 50 ng/ml rhIL-24 significantly reduced tubule formation and total tubule perimeter in comparison to untreated control cells. (B) Treatment of untreated control cells with the AKT inhibitor significantly enhanced tubule formation rates. No significant differences were observed between untreated cells and those treated with the PLCγ inhibitor. In contrast to this, a significant increase in tubule formation rates were observed between HECV cells co-treated with rhIL-24 and PLCγ inhibitor and those treated solely with rhIL-24. No significant effects were seen between HECV cells co-treated with rhIL-24 and AKT inhibitor and those treated solely with rhIL-24. Mean values±SEM are shown. *p<0.05, ***p<0.001.*

IL-24 inhibits the microtubule formation of HECV cells. A Matrigel tubule formation assay was used to examine the effect of rhIL-24 on the ability of HECV cells to form microtubules. Compared to the untreated control group, treatment with 50 ng/ml rhIL-24 resulted in a reduced total perimeter length/field of microtubules, suggesting that rhIL-24 can inhibit the microtubule formation of HECV cells (Figure 3A). Following this, small-molecule inhibitors to AKT and PLCγ signalling were used to explore the links of these pathways with the observed angiogenic effects of rhIL-24 (Figure 3B). Differential responses to these inhibitors was seen between rhIL-24-treated and -untreated groups. In untreated HECV cells the inhibition of AKT significantly enhanced the total tubule perimeter/field ($p<0.001$) whereas the inhibition of PLCγ fell just short of displaying significant effects on the total tubule perimeter/field ($p=0.054$). In contrast to this, when these inhibitors were added in conjunction to rhIL-24 treatment, no significant differences were seen following AKT inhibition and significant enhancement of total tubule perimeter was seen following PLCγ inhibition ($p<0.001$). Levels of tubule formation in the rhIL-24 + PLCγ inhibitor treatment group were similar to the levels observed in the control + PLCγ inhibitor group ($p=0.899$). Together these suggest that inhibition of PLCγ can negate the anti-angiogenic impact of rhIL-24 whereas the addition of rhIL-24 can negate the pro-angiogenic impact of AKT inhibition.

IL-24 impact on cell junction and receptor molecules. Immunofluorescent staining was used to examine the potential of rhIL-24 to effect the staining intensity and localisation of a number of cell junction molecules (Figure 4). Staining profiles were explored for Claudin-1, Claudin-5, Occludin, ZO-1 and ZO-2 in both untreated HECV cells and cells treated with 50 ng/ml rhIL-24. The representative images for both groups do not indicate any obvious

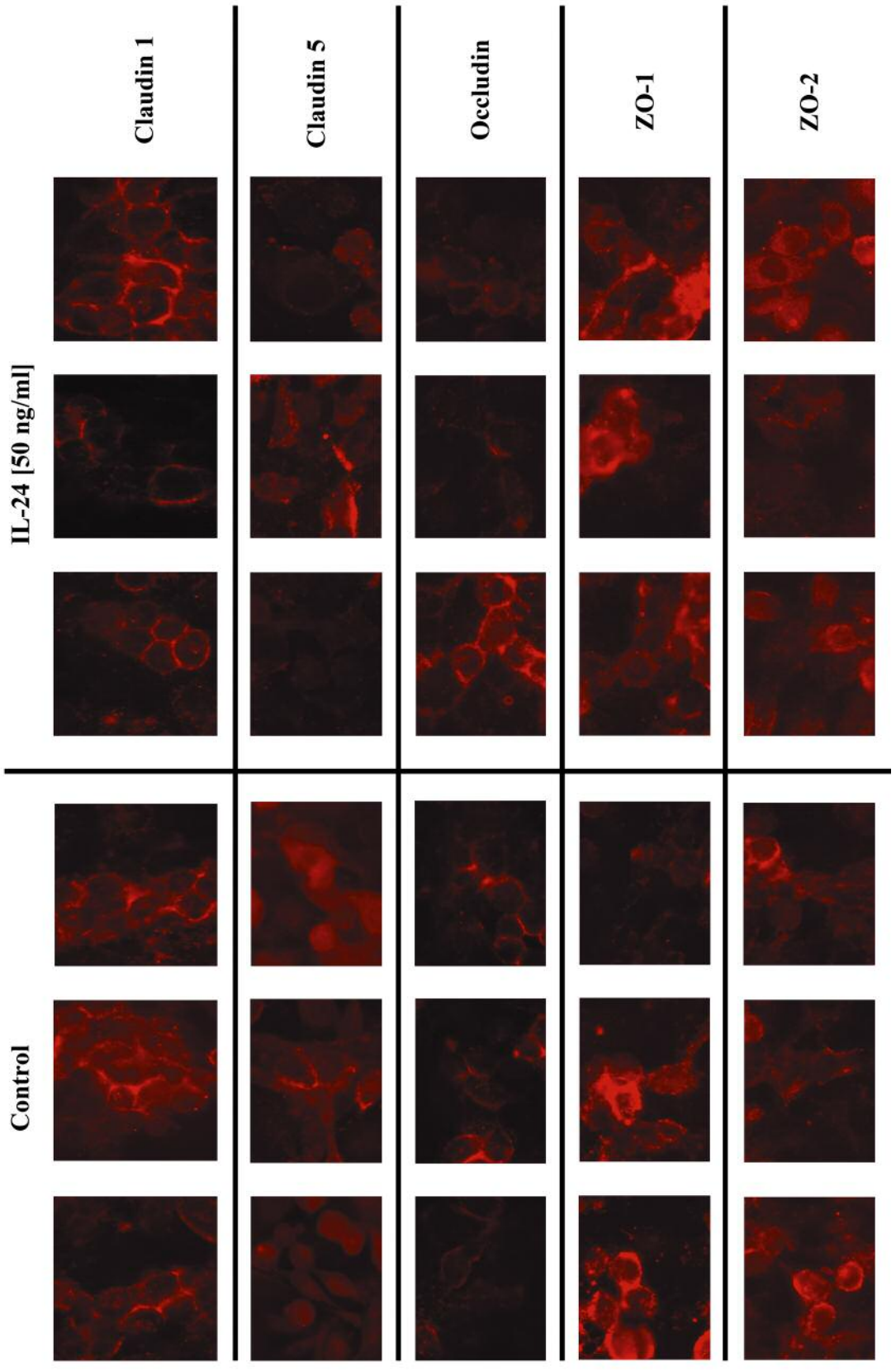

Figure 4. *Immunofluorescent staining of junctional proteins in HECV cells following rhIL-24 treatment. No substantial differences in staining intensity and localisation of the tested proteins were observed between untreated control HECV cells and those treated with 50 ng/ml rhIL-24.*

differences. In all cases, membranous staining is observed in both groups and similar levels of staining intensities are seen in both the untreated and rhIL-24-treated groups.

Discussion

Studies report that IL-24 exerts its effects through two receptors: IL-20R1/IL-20R2 or IL-22R1/IL-20R2. Expression of IL-22R1/20R2 receptor was found to be more abundant in the skin (2, 23). It has also been suggested that IL-24 can inhibit the metastatic dissemination and invasion of breast cancer *via* IL-22R (24). Herein, we found that IL-24 and its receptors are expressed in the HECV cell line.

It has been previously shown that the growth of HECV cells was reduced when exposed to IL-24 (concentration of 2.5, 25 and 250 ng/ml), and this reduction was found to be significant at the higher 250 ng/ml concentration compared to control cells (25). In the current study, similarly, no significant differences in HECV cell growth rates were observed at 50-ng/ml rhIL-24 treatment, though we did not observe any reduction in cell growth rates. Similarly, treatment with rhIL-24 did not display any substantial effects on the trans-endothelial resistance of a HECV monolayer, suggesting that this molecule may have little effects in regulating the junctional barriers of this endothelial cell line. This observation is somewhat supported by the results of the immunofluorescence experiments, which indicate that rhIL-24 treatment does not produce any substantial effects on the localisation or intensity of a number of cell junctional proteins including claudin-1, claudin-5, occludin, ZO-1 and ZO-2.

Previous studies have suggested that IL-24 had an inhibitory effect on the migration of cancer cells and keratinocytes (5, 24), and IL-24 was found to potentially slow wound healing through its inhibitory migration effect on human keratinocytes *via* an AKT-dependant pathway (15). IL-24 has also been shown to inhibit endothelial cell migration in response to VEGF (13). Our current data are somewhat in contrast to this and suggest that rhIL-24 can promote the migration of HECV endothelial cells. Our data suggest that this pro-migratory effect does not involve PLCγ signaling, although it may be related to AKT signaling as co-treatment with a small-molecule AKT inhibitor could partially, though non-significantly, reduce these pro-migratory effects. Whilst there are numerous studies demonstrating anti-migratory effects of IL-24, there is also indication that this molecule can exert pro-migratory effects as well and has been observed to promote the migratory rates of human monocytes and neutrophils (26). Hence, IL-24 appears to exert both pro-and anti-migratory effects which may be dependent on cell or tissue type or the presence of other growth factors.

IL-24 has been previously observed to have an inhibitory function on lymphangiogenesis, through a mechanism involving the decrease of the lymphangiogenic marker Prox-1 and the decreased transcript expression of lymphangiogenic factors VEGF-C and D (25) and also *via* IL-20R1/IL-20R2 or IL-22R1/IL-20R2 heterodimers (2, 27). In addition, it was reported that IL-24 played an inhibitory role on tubule formation of HUVEC cells only on tubes newly formed but not on tubes that were already established concluding that IL-24 could prevent the beginning of differentiation but couldn't overturn the differentiated endothelial cells phenotype *via* receptor IL-22R1 (13). In our study, we find that rhIL-24 (50 ng/ml) inhibits the microtubule formation and can negate the pro-angiogenic effect of AKT inhibition on HECV microtubule formation. Similarly, our data suggests links to PLCγ signalling in this respect as inhibition of this pathway with small molecule inhibitors could return levels of tubule formation to that of control cells treated with PLCγ inhibitor. This observation is in line with previous work from our laboratories conducted by Frewer *et al*., which similarly indicated an inhibitory effect of IL-24 on HECV microtubule formation (25).

In summary, IL-24 has been shown to have a variety of properties, including anti-tumour effect through apoptosis induction, depressing proliferation, inhibition of cancer cell invasion and metastasis, inhibition of cancer cell and keratinocyte migration and inhibition of lymphangiogenesis and micro-angiogenesis. IL-24 was suggested to perform a vital function in the inflammatory action in the skin stimulated by the infection of bacterial, virus or tumour cell growth, and the factors that activated IL-24 production were secreted through cells in the inflammatory reaction (14), and IL-24 played its role mainly *via* IL-20R1/IL-20R2 or IL-22R1/IL-20R2 receptors and the AKT pathway. In our study, we further elucidated the biological function of IL-24 on HECV cells. We conclude that IL-24 can increase migration and inhibit tubule formation, but does not impact on cell growth or permeability at lower concentrations. IL-24 may bring about its effects through AKT-related pathways and may also potentially be linked to PLCγ signaling.

Acknowledgements

The Authors wish to acknowledge the Albert Hung Foundation and Cancer Research Wales for supporting this work.

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Received June 22, 2015
Revised July 16, 2015
Accepted July 20, 2015