

Interleukin-8 (CXCL8) stimulates trophoblast cell migration and invasion by increasing levels of matrix metalloproteinase (MMP)2 and MMP9 and integrins α_5 and β_1

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Abstract

Interleukin-8 (IL8/CXCL8) is present in decidua and trophoblast, which also expresses the IL8 receptors, CXCR1 and CXCR2. IL8 was shown to stimulate trophoblast migration. Matrix metalloproteinase (MMP)2, MMP9, and integrins $\alpha_5\beta_1$ and $\alpha_1\beta_1$ were found to play important roles in trophoblast invasion. We hypothesized that IL8 would increase this cell migration and invasion by HTR-8/SVneo cells through the activity of MMPs and integrins. Isolated first trimester of pregnancy cytotrophoblast (CT) and HTR-8/SVneo cell line were used. Migration was studied by monolayer wounding test, and invasion by Matrigel invasion test. The effects of IL8 on MMPs and integrin subunit expression were determined in HTR-8/SVneo cells by gelatin zymography and western blot respectively. The results that were obtained showed that exogenous IL8 stimulated HTR-8/SVneo cell migration and invasion. MMP2 and MMP9 levels were stimulated to 182% ($P < 0.01$) and 134% ($P < 0.01$) respectively. Integrin α_5 expression was increased to 119% ($P < 0.05$) and integrin β_1 expression to 173% ($P < 0.001$) of the control values. The data that were obtained show for the first time the sensitivity of the HTR-8/SVneo cells, in addition to isolated first trimester CT, to IL8. Exogenous IL8/CXCL8 increased trophoblast cell migration and invasion, which may be partly attributable to stimulation of MMP2 and MMP9 levels and an increase in integrins. HTR-8/SVneo cell viability and proliferation were also increased.

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Introduction

Extravillous trophoblast (EVT) cells are specific cells of the placenta that can invade into the decidual stroma and spiral arteries of the uterus. Invasion of the uterine decidua and inner third of the myometrium by EVT cells is a step that is crucial for establishing a successful pregnancy (Pijnenborg *et al.* 1983). During this process, trophoblast cells undergo a change in integrin phenotype acquiring integrins $\alpha_5\beta_1$ and $\alpha_1\beta_1$ (Damsky *et al.* 1992). The invasive properties of the EVT are linked to their ability to degrade the extracellular matrix (Fisher *et al.* 1985) by secreting proteolytic enzymes (reviewed by Cohen *et al.* (2006)). Matrix metalloproteinases-2 and -9 (MMP2 and MMP9) are particularly relevant for this process (Lala & Graham 1990, Librach *et al.* 1991). Various autocrine and paracrine factors have been suggested to modulate trophoblast function (reviewed by Salamonsen *et al.* (2007)). Many cytokines are produced by both trophoblast and diverse decidual cell types, and some, such as tumor necrosis factor- α (TNF; Bauer *et al.* 2004), interleukin-1 (IL1; Librach *et al.* 1994), interferon- γ (Lash *et al.* 2006), leukemia inhibitory factor (LIF;

Poehlmann *et al.* 2005), IL6 (Jovanović & Vićovac 2009), and transforming growth factor- β (TGFB; Caniggia *et al.* 1999, Lash *et al.* 2005), are implicated in the regulation of trophoblast invasion.

In the first trimester of pregnancy, when trophoblast invasion is most extensive, decidualized endometrium is composed of different cytokine-producing immune and non-immune cell types (Starkey *et al.* 1988). One of these cytokines is IL8 (CXCL8), a proinflammatory molecule belonging to the CXC chemokine subfamily. Within the uterus, the presence of IL8 has been documented in endometrial (Arici *et al.* 1993) and decidual (Saito *et al.* 1994) epithelial and stromal cells, decidual natural killer (NK) cells (Hanna *et al.* 2006), decidual macrophages exposed to trophoblast (Fest *et al.* 2007), and decidual CD8⁺ T lymphocytes (Scaife *et al.* 2006). This 8 kDa non-glycosylated protein is produced by many cell types, including monocytes, lymphocytes, granulocytes, fibroblasts, endothelial cells, bronchial epithelial cells, keratinocytes, hepatocytes, mesangial cells, and chondrocytes (reviewed by Iizasa & Matsushima (2000)). IL8 is a potent neutrophil chemoattractant (Harada *et al.* 1994) and stimulator of neutrophil

transendothelial migration (Huber *et al.* 1991). It also induces angiogenesis (Koch *et al.* 1992) and inhibits proliferation of myeloid progenitor cells (Sanchez *et al.* 1998). Trophoblast and placental macrophages constitutively produce IL8, the concentration of which is highest at term (Shimoya *et al.* 1992). IL8 mRNA and protein were also localized in cytotrophoblast (CT), syncytiotrophoblast, and Hofbauer cells of the placenta (Saito *et al.* 1994). IL8 binds to two receptor types, CXCR1 and CXCR2. The former has been demonstrated on EVT (Hanna *et al.* 2006), isolated CT, and the BeWo cell line (Tsui *et al.* 2004). Villous cytotrophoblast (vCT) cells were shown to express mRNA for both CXCR1 and CXCR2 (Hirota *et al.* 2009). IL8 from decidual NK cells (Hanna *et al.* 2006) and endometrial epithelial cells (Hirota *et al.* 2009) was found to promote EVT migration, and to stimulate survival of vCT cells *in vitro* (Hirota *et al.* 2009). The aim of this study was to determine the suitability of the HTR-8/SVneo cell line, derived from the first trimester EVT, as a model for IL8-induced effects on the invasive trophoblast. We hypothesized that IL8 would 1) increase cell migration and invasion of HTR-8/SVneo cells, 2) increase levels of MMP2 and MMP9, as well as the expression of integrins, and 3) increase cell proliferation and/or survival.

Results

Even though IL8 was reported to influence trophoblast cell migration (Hanna *et al.* 2006), its effect on the human trophoblast has not been studied using the HTR-8/SVneo cell line. Earlier studies showed that HTR-8/SVneo immortalized first trimester of pregnancy trophoblast cells produced IL8 at concentrations of 0.06–1 ng/ml in 24 h depending on culture conditions (Svinarich *et al.* 1996, Chou *et al.* 2006). Here, we confirmed that this cell line produces IL8, as weak to moderate cytoplasmic staining was detected by immunocytochemistry in both CT (identified by CK7 staining, Fig. 1A) and HTR-8/SVneo cells (Fig. 1B). Isolated EVT was found to express CXCR1 protein (Hanna *et al.* 2006). In addition to isolated trophoblast (Fig. 1C), we detected the CXCR1 in HTR-8/SVneo cells as well (Fig. 1D). The same receptor was detected on both cell types by flow cytometry (Fig. 2). Flow cytometric analysis provided data that 90% of isolated CT (Fig. 2A) and 99% of HTR-8/SVneo cells (Fig. 2B) were positive.

Under some conditions, the invasive trophoblast *in vivo* may be exposed to considerable amounts of IL8 from one or more cell types that produce it within the decidua. Therefore, we wished to establish whether additional exogenous IL8 may influence trophoblast invasion and migration. The potentially effective concentrations of IL8 for HTR-8/SVneo cells were in the lower nanogram range of IL8 (0.5–10 ng/ml) in cell migration tests (Fig. 3) at 3 and 24 h of culture. The data had a bell-shaped curve, with all tested concentrations (0.5, 1, 5, and 10 ng/ml) having stimulatory effects on

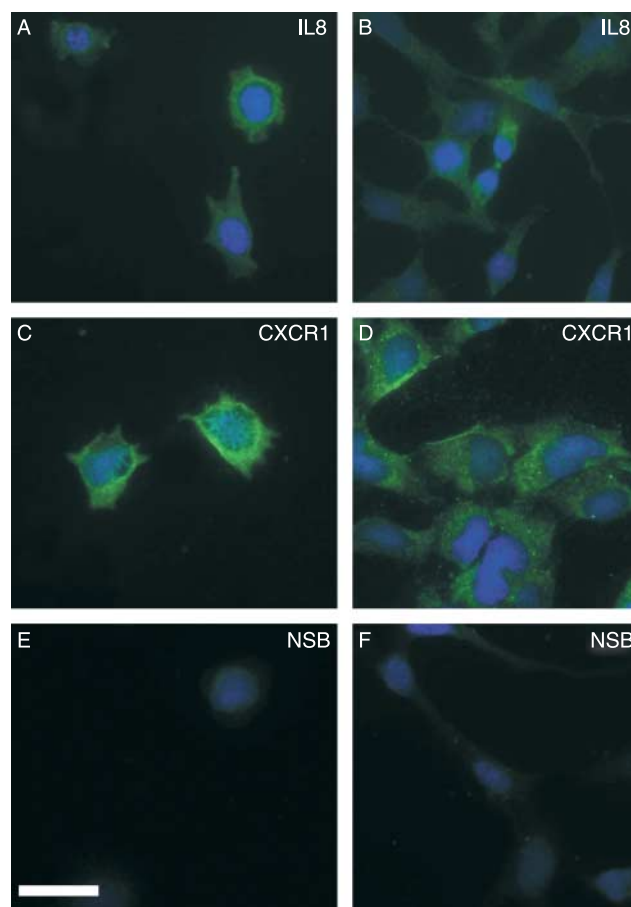


Figure 1 Expression of IL8 and IL8 receptor CXCR1 by trophoblast cells. Immunocytochemical detection of IL8 (A and B) and CXCR1 (C and D) in cytotrophoblast (A, C, and E) and HTR-8/SVneo cells (B, D, and F). Both cell types are stained for IL8 (fluorescence in A and B) and CXCR1 (fluorescence in C and D). Non-specific staining (NSB, isotype-matched control IgG, E and F), and nuclei stained with DAPI (blue), scale bar = 20 μ m.

HTR-8/SVneo cell migration (124, 166, 167, and 131% of the control values respectively) after 3 h of culture (significant at $P < 0.05$, $P < 0.001$, $P < 0.001$, and $P < 0.05$ respectively; Fig. 3A). After 24 h of culture, the effects of IL8 were less pronounced, but were stimulatory for 1 and 5 ng/ml (127%, $P < 0.001$, and 125%, $P < 0.001$, of the control values respectively; Fig. 3B). The time course of this effect for a representative field is shown in the photomicrograph in Fig. 3C.

In migration and invasion experiments, function-blocking anti-IL8 or non-immune immunoglobulins were added to culture media in order to assess the influence of endogenous IL8 on cell migration and invasion. Addition of function-blocking anti-IL8 antibodies at 1 μ g/ml had no effect on cell migration after 3 h of culture (Fig. 3A), but after 24 h of culture, anti-IL8 antibodies decreased cell migration to 90% ($P < 0.01$) of the control (Fig. 3B), while non-immune IgG at 1 μ g/ml had no effect on cell migration. The stimulation by exogenous IL8 at 5 ng/ml was decreased

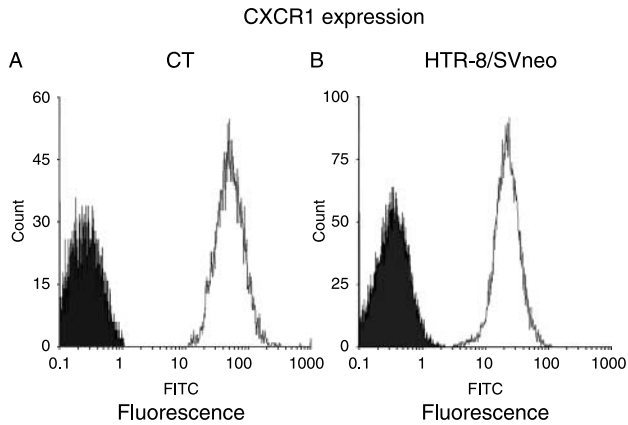


Figure 2 Expression of CXCR1 in isolated cytotrophoblast (CT) (A) and HTR-8/SVneo cells (B) measured cytofluorometrically. The x-axis indicates fluorescence intensity measured on a log10 scale, and the y-axis indicates event counts on a linear scale. Specific staining is shown as white histograms, and non-specific staining (isotype-matched control IgG) as black histograms. A representative experiment of three for isolated CT is shown, and of five for HTR-8/SVneo cells is shown.

to values that were not significantly different from the control values when function-blocking anti-IL8 antibodies at 5 µg/ml were added (Fig. 3A and B).

In the Matrigel invasion assay, IL8 at 5 ng/ml was found to increase invasion significantly both by freshly isolated CT (Fig. 4A) to 175% ($P < 0.01$) of the control and by HTR-8/SVneo cells (Fig. 4B) to 150% of the control ($P < 0.001$). With function-blocking anti-IL8 antibodies (1 µg/ml), unstimulated HTR-8/SVneo cell invasion was 80% ($P < 0.05$; Fig. 4B) and isolated CT invasion was 90% of the control value, but this slight inhibition was not statistically significant. Non-immune IgG had no effect on cell invasion in either cell type. The addition of anti-IL8 at 5 µg/ml to the media with recombinant IL8 at 5 ng/ml decreased the invasion to values that were not significantly different from the control values (Fig. 4A and B).

To elucidate the potential effector molecules involved in IL8-induced cell migration and invasion, MMP2 and MMP9 gelatinase levels were studied using gelatin zymography. Densitometric analysis revealed that IL8 stimulated pro-MMP2 level to 182% of the control values (Fig. 5A, $P < 0.01$) and pro-MMP9 level to 134% (Fig. 5B, $P < 0.01$) of the control values respectively.

Integrin subunit α_5 , α_1 , and β_1 expression in HTR-8/SVneo cells was examined by western blot analysis of lysates of cells treated with IL8. Densitometry showed specific bands of ~150, 130, and 130 kDa for integrins α_5 , β_1 , and α_1 respectively. The bands for α_5 and β_1 integrin subunits were more intense in IL8-treated HTR-8/SVneo cells. All intensities were normalized to actin band intensity, and were compared. Integrin subunits α_5 and β_1 were increased to 119% (Fig. 6A, $P < 0.05$) and 173% of the control values (Fig. 6B, $P < 0.001$), while integrin α_1 expression was not affected by IL8 (Fig. 6C).

The possibility that IL8 may influence trophoblast cell proliferation and survival was tested in the HTR-8/SVneo cell model. In the 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide; thiazolyl blue (MTT) test, viable cell numbers detected in treated versus untreated cultures showed that IL8 (5 ng/ml) induced an increase to 112% of the control value ($P < 0.001$) after 24 h of culture and to 115% ($P < 0.001$) after 48 h (Fig. 7C).

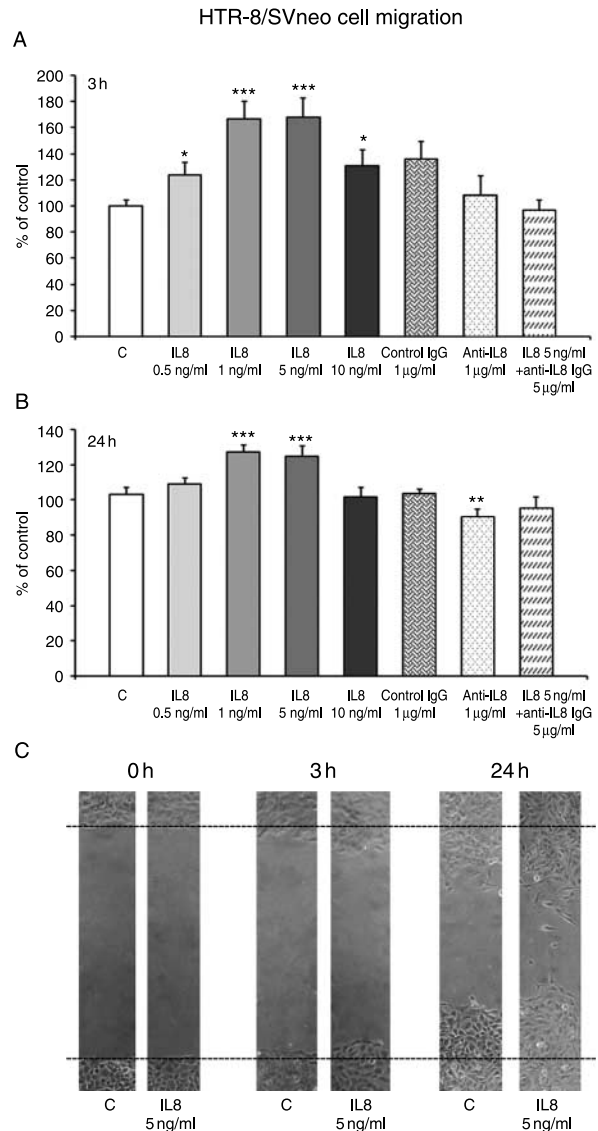


Figure 3 IL8 induced stimulation of HTR-8/SVneo cell migration. Time and concentration dependence of the IL8 effect on HTR-8/SVneo cell migration and neutralization of IL8 with specific function-blocking antibody. The effect of nanogram concentrations (0.5, 1, 5, and 10 ng/ml) of IL8 and anti-IL8 antibody at 1 or 5 µg/ml after 3 h (A) and 24 h of culture (B). The distances were measured using an electronic grid (0.1 × 0.1 mm²). Photomicrograph of cell migration after 0, 3, and 24 h for control and IL8-treated cells (5 ng/ml), a representative field (C). Data (from four experiments, two replicate wells, and three areas measured per well) are expressed as the percentage of the untreated control, with values given as mean ± S.E.M., and statistical significance given as * for $P < 0.05$; ** for $P < 0.01$; and *** for $P < 0.001$.

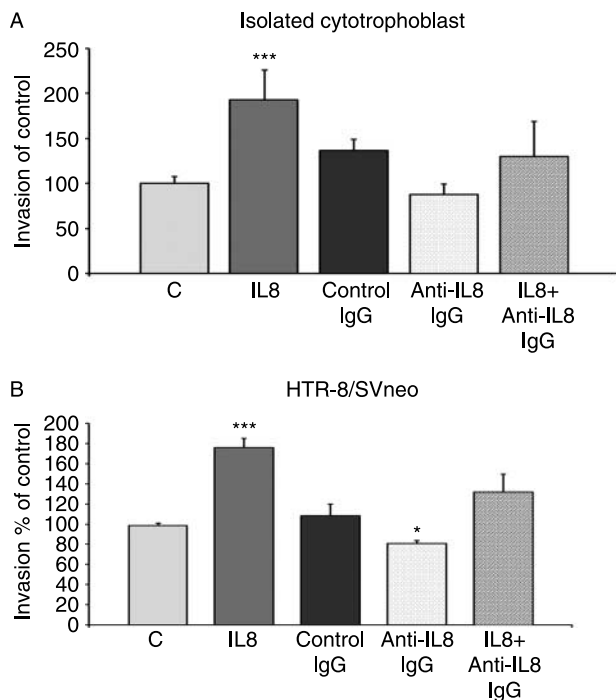


Figure 4 IL8 induced stimulation of trophoblast invasion. Effects of IL8 (5 ng/ml), function-blocking anti-IL8 antibodies (1 or 5 µg/ml), and non-immune IgG (1 µg/ml) on cell invasion by (A) freshly isolated cytotrophoblast from the first trimester placenta and (B) HTR-8/SVneo cell line. Cells on the underside of the filters and the pores occupied were counted after 20 h of culture for isolated cytotrophoblast and after 24 h for HTR-8/SVneo cells. The data are expressed as the percentage of the pores occupied in the control, with values given as mean \pm s.e.m. Differences were significant at $P < 0.01$ for primary trophoblast (four experiments and two replicates) and at $P < 0.001$ for HTR-8/SVneo cell invasion (six experiments and two replicates) treated with IL8. Treatment with anti-IL8 antibodies had an inhibitory effect on HTR-8/SVneo cell invasion ($P < 0.05$), and a slight but non-significant effect on isolated cytotrophoblast invasion. Non-immune IgG had no effect on invasion by either cell type.

The influence of IL8 on cell proliferation was assessed by immunocytochemistry for the Ki67 antigen (Fig. 7A). At 5 ng/ml, IL8 increased the proportion of Ki67-positive HTR-8/SVneo cells to 146% of the control value (Fig. 7B, $P < 0.001$). Using the M30 antibody to the caspase-cleaved fragment of cytokeratin-18, the percentage of apoptotic HTR-8/SVneo cells was found to be very low and not altered by treatment with IL8 at 5 ng/ml (Fig. 7D).

Discussion

Our results provide evidence that IL8 stimulates the ability of the extravillous CT to migrate and invade through the extracellular matrix *in vitro*, which is accompanied by increases in MMP2 and MMP9 proform levels and integrins α_5 and β_1 . At the fetal–maternal interface, multiple sources of IL8 have been identified that include CT, syncytiotrophoblast, fetal macrophages,

decidual stromal cells, decidual lymphocytes, and endometrial epithelial and gland cells (Saito *et al.* 1994, Hirota *et al.* 2009). Among the cells isolated from the decidua, high levels of IL8 were secreted by activated human decidual CD8⁺ T lymphocytes (Scaife *et al.* 2006), and IL8 was produced by CD14⁺ monocytes (Fest *et al.* 2007) as well as by decidual NK cells (Hanna *et al.* 2006). This cytokine modulates multiple biological functions in CXCR1- and CXCR2-expressing cells, among which CXCR1 has been detected on invasive trophoblast (Hanna *et al.* 2006). We present data that both isolated CT and HTR-8/SVneo cells used in functional tests here also express the CXCR1. Cell migration and invasion experiments demonstrated the functionality of the receptors in both cell types. We have tested the hypothesis that IL8 regulates trophoblast migration and invasion in a paracrine and possibly autocrine manner. A range of other chemokine receptors have been found in trophoblast (Hanna *et al.* 2006, Hannan *et al.* 2006), and some of their ligands were also shown to promote trophoblast migration (Hannan *et al.* 2006). Thus, a more complex picture is likely to emerge regarding the role of chemokines in trophoblast migration. Unstimulated HTR-8/SVneo cells have already been shown to produce IL8 at the mRNA and protein levels (Svinarich *et al.* 1996, Chou *et al.* 2006), and this was increased by treatment with lipopolysaccharide (Svinarich *et al.* 1996) and was augmented further by pre-exposure to CRH (Wang *et al.* 2007). The autocrine action of IL8 was tested by neutralization in HTR-8/SVneo cells. Our data show that blocking endogenous IL8 in unstimulated trophoblast mildly decreased migration in HTR-8/SVneo cells, which is likely to reflect low IL8 levels in unstimulated HTR-8/SVneo cells (Svinarich *et al.* 1996, Chou *et al.* 2006).

The stimulation of migration by IL8 shown here for HTR-8/SVneo cells is in keeping with previous results for normal trophoblast in culture (Hanna *et al.* 2006).

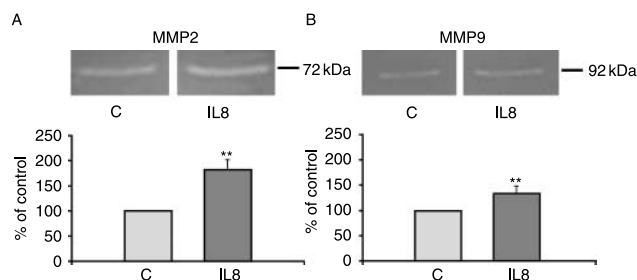


Figure 5 The effect of IL8 (5 ng/ml) on the MMP2 (A) and MMP9 (B) proform protein levels in HTR-8/SVneo cell culture supernatants in the gelatin zymography assay. Gelatinase levels were semi-quantitated by densitometric analysis of the corresponding bands using the ImageMaster TotalLab v2.01 programme. Data are expressed as the percentage of untreated control with values given as mean \pm s.e.m. (three experiments and two replicates). Statistical significance is given as ** for $P < 0.01$.

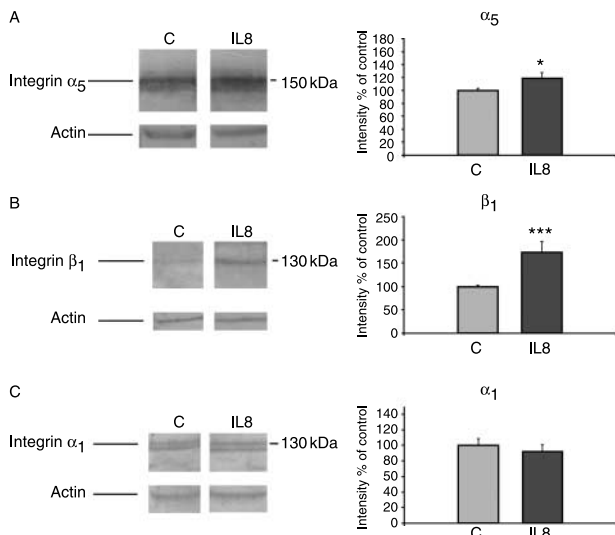


Figure 6 Western blot analysis of integrin α_5 , β_1 , and α_1 subunit expression in control (C) and IL8-treated (5 ng/ml) HTR-8/SVneo cells. Proteins in cell lysates were resolved by SDS-PAGE on 10% gel under reducing conditions, and were transferred to nitrocellulose membrane. Membranes with immobilized proteins were probed with anti-integrin α_5 (A), anti-integrin β_1 (B), and anti-integrin α_1 (C) subunit-specific antibodies. The charts show the densitometric analysis of specific bands using the ImageMaster TotalLab v2.01 programme ($n=6$, from three cultures). Treatment with IL8 (5 ng/ml) induced significant increases in integrins α_5 and β_1 , $P<0.05$ and $P<0.001$ respectively, but it had no effect on α_1 subunit expression. The intensities normalized to actin are expressed as the percentages of untreated control values, and are given as mean \pm s.e.m. Statistical significance is shown as * for $P<0.05$ and *** for $P<0.01$.

In contrast to the recombinant IL8 used here, Hanna *et al.* used NK cell supernatant, potentially containing additional active substances, that induced stimulation of migration. We also found enhanced invasion of Matrigel by HTR-8/SVneo cells, and isolated trophoblast exposed to exogenous IL8. In addition, endometrial stromal cells in co-culture with trophoblast were found to up-regulate *IL8* most among other genes (Popovici *et al.* 2006), which suggests the relevance of IL8 for the process of trophoblast invasion. Consistent with our data, IL8 acting in a paracrine fashion qualifies as a factor contributing to permissiveness of the uterine tissues for trophoblast invasion. It has been proposed that IL8 may function as part of the signaling loop between the implanting embryo and the permissive maternal environment, since fetal IL1 stimulated IL8 in endometrial epithelial cells, which in turn was found to stimulate migration and survival of vCT cells *in vitro* (Hirota *et al.* 2009). Decidual NK cell-derived IL8 increased migration/invasion by trophoblast (Hanna *et al.* 2006). Since endothelial cells also produced IL8 under flow stress (Cheng *et al.* 2007), IL8 signaling might be relevant for both interstitial and endovascular trophoblast invasion.

It has been suggested that endometrial IL8 can act as a survival factor for trophoblast (Hirota *et al.* 2009). Our data are supportive of this possibility in the case of the immortalized cell line HTR-8/SVneo, since viable cell numbers were increased upon treatment with IL8. When treated HTR-8/SVneo cells were further analyzed by immunocytochemistry, no effect on apoptosis was observed, but IL8 acted as a proliferation-increasing factor for this EVT cell line. A similar influence of IL8 on

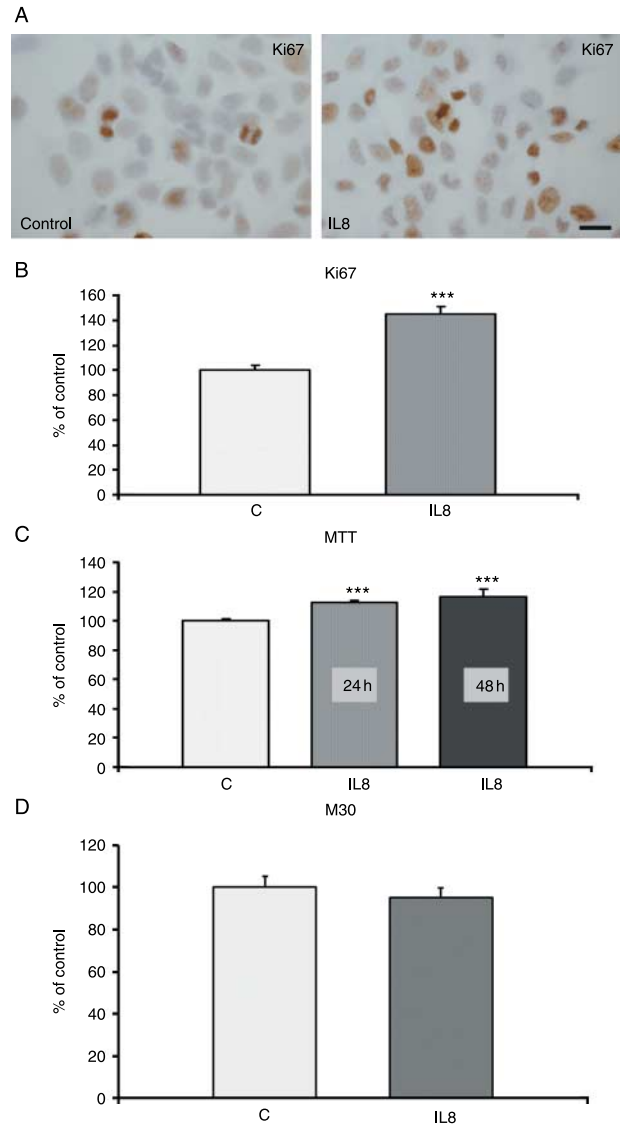


Figure 7 The effect of IL8 on HTR-8/SVneo cell proliferation. Expression of the proliferation marker, Ki67, in control and IL8-treated (5 ng/ml) cell cultures (A). Effects of IL8 on the number of Ki67-positive cells after 72 h of culture (B, three experiments, 30 fields counted from each, and total > 3000 cells). The effect of IL8 on viable cell numbers in the MTT test (C, five experiments and 12 replicates in each) and on apoptosis as evidenced by immunostaining for M30 (D, three experiments, 30 fields counted from each, and total > 3000 cells). Data are expressed as the percentage of untreated control values given as mean \pm s.e.m. Statistical significance is shown as *** for $P<0.001$. Scale bar = 10 μ m.

cell proliferation was shown for tumor cells, because numbers of proliferating cells were reduced when IL8 was silenced (Merritt *et al.* 2008). This effect of IL8, however, may not be relevant for non-proliferative EVT.

Cellular invasion requires proteolytic degradation of extracellular matrix molecules. The MMP system is one of the well-documented effector mechanisms important for trophoblast invasion (Librach *et al.* 1991). The MMPs comprise a large family of enzymes that includes collagenases, stromelysins, and gelatinases, and trophoblast cells are a major source of gelatinases, MMP2 and MMP9 (Cohen *et al.* 2006). The effect of IL8 on cellular protease levels has not been studied previously in trophoblast cells, but in other systems, effects of IL8 have been shown to depend on MMP activity (Gálvez *et al.* 2005). Thus, the availability of IL8 for endothelial cells was found to influence proliferation, apoptosis, migration, and production of MMP2 (Li *et al.* 2003). In cancer models, blocking IL8 induced tumor reduction involving down-regulation of MMP2 and MMP9 activity through modulation of nuclear factor- κ B expression and transcriptional activity (Mian *et al.* 2003). In pancreatic cancer cell lines, IL8 induced MMP2 and MMP9 activity without increasing proliferation (Kuwada *et al.* 2003). Other cytokines that affect trophoblast invasiveness have also been found to modulate MMP2 and MMP9 activity, such as IL1 (Librach *et al.* 1994), IL6 (Meisser *et al.* 1999), and TGF β (Lash *et al.* 2005).

It is well established that both the integrin switch in trophoblast differentiation along the extravillous invasive pathway and the activity of specific MMPs are critical for the formation of a fully functional fetal–maternal interface (Damsky *et al.* 1994). Failure to express adequate integrins has been associated with reduced invasion *in vitro* (Genbacev *et al.* 1996) and pathological conditions with impaired trophoblast invasion *in vivo*, such as pre-eclampsia (Zhou *et al.* 1993). As subunits forming fibronectin and laminin/collagen receptors, we studied the integrin proteins α_1 , α_5 , and β_1 because the involvement of integrins in IL8 stimulation of trophoblast migration has not been investigated so far. Increases in α_5 and β_1 integrin proteins were found in HTR-8/SVneo cells treated with IL8. Other cytokines and growth factors influencing trophoblast invasion, such as epidermal growth factor or IL6, were also reported to affect integrin expression (Leach *et al.* 2004, Jovanović & Vićovac 2009).

It can be concluded that the HTR-8/SVneo cell line expresses functional CXCR1 for IL8, which acts to increase cell migration and invasion, in the manner described for normal EVT. We have shown for the first time that this stimulation is accompanied by increases in pro-MMP2 and pro-MMP9 and integrins α_5 and β_1 . The overall context of IL8-related signaling within the gravid uterus is consistent with the hypothesis that paracrine IL8 contributes to permissiveness of the uterine tissues for trophoblast invasion.

Materials and Methods

Reagents and antibodies

RPMI 1640 medium and FCS were obtained from PAA Laboratories (Linz, Austria), Matrigel and collagen type I were obtained from BD Biosciences (Bedford, MA, USA), and IL8 was obtained from R&D Systems (Oxford, UK). The following antibodies were used: mouse anti-human IL8 with IL8-neutralizing activity (clone 6217), anti-IL8 receptor (clone 42 705), anti-integrin α_5 (R&D Systems), rabbit anti-integrin β_1 (Chemicon, Temecula, CA, USA), mouse anti-integrin α_1 (Abcam, Cambridge, UK), mouse anti-Ki67 and mouse anti-CK7 (Dako, Glostrup, Denmark), mouse anti-M30 (Roche), mouse anti-cytokeratin-18, and rabbit anti-actin (Sigma). DMEM/F12, trypan blue, gentamycin, antibiotic–antimycotic solution, acrylamide, *N,N'*-methylene-bis-acrylamide, *N,N,N',N'*-tetramethylethylenediamine, Ponceau S, glycine, protease inhibitor cocktail, and MTT were obtained from Sigma Chemical Company. SDS-PAGE protein standards were obtained from Bio-Rad. Non-immune mouse IgG, biotinylated horse anti-mouse and goat anti-rabbit IgG, avidin–biotinylated peroxidase complex (ABC), and diaminobenzidine (DAB) substrate kit for peroxidase were obtained from Vector Laboratories (Burlingame, CA, USA). Anti-mouse IgG antibody Alexa Fluor 488 and Prolong Gold antifade reagent with DAPI were obtained from Molecular Probes (Invitrogen). Tris and 2-mercaptoethanol were obtained from ICN Biomedicals, Inc. (Aurora, OH, USA). Protran nitrocellulose transfer membrane was obtained from Schleicher & Schuell BioScience GmbH Whatman Group (Dassel, Germany). All other reagents were of the best commercial grade available.

Antibody dilutions for immunocytochemistry were 1:6000 for anti-cytokeratin-18, 1:75 for anti-cytokeratin-7, 1:10 for anti-IL8 and anti-IL8R, 1:50 for anti-Ki67, 1:25 for anti-M30, 1:200 for biotinylated horse anti-mouse IgG, and 1:1000 for Alexa Fluor 488 anti-mouse IgG. For western blot analysis, dilutions were 1:100 for anti-integrin α_5 , 1:500 for anti-integrin β_1 , 1:20 for anti-integrin α_1 antibody, and 1:2000 for anti-actin. Biotinylated horse anti-mouse and goat anti-rabbit IgG were diluted 1:750 for western blot analysis.

Isolation of CT and cell culture

HTR-8/SVneo cells were kindly provided by Dr Charles H Graham (Queen's University, Kingston, Ontario, Canada). This cell line was obtained from human first trimester placenta explant cultures immortalized by SV40 large T antigen (Graham *et al.* 1993, Irving *et al.* 1995). Primary CT cells were isolated from the first trimester placentas from legal abortions (6–12 weeks) undertaken for non-medical reasons at the Institute of Obstetrics and Gynecology, Clinical Center of Serbia, Belgrade, Serbia, in accordance with the local ethical standards as reported previously (Librach *et al.* 1991). Briefly, placentas were washed in sterile 0.05 M PBS, pH 7.2 (s-PBS), containing gentamycin and were subjected to two 25-min cycles of digestion with 0.25% trypsin (v/v) and 0.2 mg/ml DNase I in s-PBS containing 5 mmol/l MgCl₂. Cells released from each step were pooled and filtered through a 40- μ m nylon sieve (Nylon Bolting Cloth, Locketex, Warrington, UK),

resuspended in 20% Percoll (Pharmacia), and subjected to discontinuous Percoll gradient centrifugation. The trophoblast-containing cell suspension from the middle gradient layer was washed in s-PBS containing 0.1% BSA (w/v) and was plated at 1×10^6 /ml in DMEM/F12, supplemented with 10% FCS. Cells were identified as trophoblast by immunocytochemical staining for cytokeratin-18 and cytokeratin-7. In all experiments, more than 90% of the cells were cytokeratin positive. The procedure employed to isolate CT did not involve mincing of placentas, and the cytokeratin-18-positive count was in good agreement with that for cytokeratin-7. Since the cell preparations also contained a small percentage of CD45-positive cells (<6%), these cells were removed using immunomagnetic beads prior to IL8 receptor evaluation by FACS as described below.

Primary CT was cultured in DMEM/F12 supplemented with 10% FCS (v/v), and HTR-8/SVneo cells were cultured in RPMI 1640 supplemented with 5% FCS (v/v) with antibiotic–antimycotic solution. For SDS-PAGE, HTR-8/SVneo cell lysates were prepared after 24 h of incubation with or without IL8 (5 ng/ml). Cells from tissue culture flasks were trypsinized, washed twice in s-PBS, counted, and lysed in a sample buffer containing a protease inhibitor cocktail (6×10^6 cells/ml). Insoluble material was removed by centrifugation (1600 g for 5 min at 4 °C). Samples were heated for 5 min in a boiling water bath.

For immunocytochemical analysis, isolated CT and HTR-8/SVneo cells were cultured on glass cover slips. CT was cultured for 18 h, and HTR-8/SVneo cells were cultured until ~80% confluence was reached (72 h) at 37 °C in a moist atmosphere of air with 5% CO₂. Cover slips were then rinsed twice with s-PBS and were fixed with ice-cold acetone–methanol (1:1) for 10 min. Cover slips were kept frozen until staining.

For the MTT test, HTR-8/SVneo cells were seeded in 96-well plates in 100 µl of medium at 2×10^4 cells/well. Cells were allowed to adhere overnight in a humidified chamber with 5% CO₂ at 37 °C. Cells were then carefully rinsed with s-PBS and were challenged with IL8 (5 ng/ml) dissolved in the medium to a total culture volume of 200 µl. After 24 h, the cells were carefully rinsed twice with warm s-PBS.

For gelatin zymography, 1×10^5 HTR-8/SVneo cells were seeded in 300 µl RPMI 1640 with 5% FCS (v/v) per well in 24-well plates. Cells were incubated at 37 °C in a moist atmosphere of air with 5% CO₂ until close to confluence. They were then carefully rinsed twice with warm s-PBS, and 200 µl of serum-free RPMI 1640 without or with IL8 (5 ng/ml) were added. Cells were incubated for another 24 h. Culture media were then collected and centrifuged for 5 min at 700 g to remove any cells, and the protein concentrations were determined using the BCA assay (Pierce Biotechnology, Rockford, IL, USA).

Immunocytochemistry

Cell cultures were washed with PBS, air-dried, and fixed with ice-cold acetone–methanol. Non-specific binding was blocked for endogenous peroxidase activity with 1% hydrogen peroxide for 30 min, and non-specific binding of proteins was blocked with 1% casein in PBS for 20 min at room

temperature (RT). The cells were then incubated with the primary anti-cytokeratin-18 antibody for 1 h at RT. This was followed by incubation with biotinylated horse anti-mouse IgG secondary antibody for 30 min and with ABC for another 30 min. Between steps, cells were washed with PBS. Reaction was visualized using DAB as the chromogen. Negative controls were performed routinely. Omission of the primary antibody and use of non-immune serum in place of specific antibody resulted in the complete absence of staining. Slides were then mounted and examined using a Reichert-Jung microscope with Leica DC150 Digital Camera System (Wetzlar, Germany). Cell purity of the isolated trophoblast determined by immunostaining with cytokeratin-18 was >90%.

HTR-8/SVneo cells were stained for Ki67 antigen as a marker of cell proliferation and for M30 as a marker of cell apoptosis using the procedure described above. Reaction was visualized using DAB as the chromogen. The nuclei were visualized by hematoxylin staining. Slides were then mounted and examined using a Reichert-Jung microscope with Leica DC150 Digital Camera System. Ki67- and M30-positive cells were counted on cover slips from three different experiments.

Isolated CT and HTR-8/SVneo cells on cover slips were stained using mouse anti-hIL8 and anti-hIL8R antibody and anti-mouse IgG antibody Alexa Fluor 488. Cells were first stained for CK-18 using biotinylated anti-mouse IgG antibodies followed by ABC with DAB as the chromogen to confirm its identity as described above. The cells were then extensively rinsed with PBS and blocked for non-specific binding with 1% casein in PBS (w/v) for 20 min. They were incubated with anti-hIL8 and anti-hIL8R antibodies overnight at 4 °C, and then with anti-mouse IgG antibody Alexa Fluor 488 for 30 min. Slides were mounted with Prolong Gold antifade reagent with DAPI and examined using a Carl Zeiss Axio Imager 1.0 microscope (Jena, Germany), with a Canon A640 Digital Camera System (Tokyo, Japan).

Flow cytometry

Flow cytometry was used to evaluate the expression of CXCR1 on HTR-8/SVneo and isolated trophoblast cells. Magnetic beads coated with antibody to CD45 were used to deplete bone marrow-derived cells from the isolated CT cell suspension. Dynabeads – M-280 sheep anti-mouse IgG (Invitrogen) were coated with mouse anti-CD45 MAB (1:100; Serotec, Oxford, UK) according to the manufacturer's instructions. The coated beads were stored in PBS/0.1% BSA at 4 °C until use. Isolated cell suspensions were incubated with coated beads for 1 h at 4 °C with rotation. Beads with attached CD45-positive cells were removed magnetically. The resulting CT cells were washed four times with PBS/0.1%BSA, and the purity was estimated by cytopins stained with anti-CD45 and anti-CK7 antibodies. The purity of the stained cells was >98%. Purified CT cells (8×10^5 cells/tube) or HTR-8/SVneo cells (8×10^5 cells/tube) were washed twice with cold PBS₂ (PBS, 2% FCS, and 0.01% sodium azide), and were permeabilized with fixation/permeabilization concentrate diluted in fixation/permeabilization diluent, 1:4 (eBioscience, San Diego, CA, USA), overnight at 4 °C. Cells were washed twice in permeabilization buffer (PB), diluted in deionized water (1:10),

and incubated with anti-CXCR1 antibody for 45 min at 4 °C. After incubation and subsequent washing with PB, cells were stained for 30 min with Alexa Flour 488 goat anti-mouse IgG diluted 1:1000 in PB with 5% goat serum. Control cells were incubated with non-immune mouse IgG as the primary antibody. Labeled cells were fixed with 4% formalin, and were analyzed on an EPICS XL-MCL flow cytometer (Coulter, Krefeld, Germany).

Cell wounding and migration assay

The effect of IL8 and anti-IL8 antibodies on HTR-8/SVneo cell migration was investigated as follows: 5×10^5 HTR-8/SVneo cells were plated in 6-well plates in 2 ml of medium and incubated at 37 °C in 5% CO₂ until confluence. Cells were then scraped off with a sterile pipette tip and rinsed with s-PBS twice. Medium (2 ml) without or with IL8 at 0.5, 1, 5, and 10 ng/ml and without or with function-blocking anti-IL8 or non-immune IgG at 1 µg/ml was then added. The pre-selected fields were photographed at 0 point after 3 and 24 h. The width of the denuded area was measured using an electronic grid, and the distances crossed by the cells were determined. The mean of the controls was set to 100%, and the data are expressed as the percentage of the control values. The experiment was repeated four times in duplicate.

Cell invasion assay

Transwell invasion assays were conducted in 24 well-fitted wells with membranes (polyethylene terephthalate, track-etched membrane of 6.4 mm diameter, with 8 µm porosity, Falcon, BD Labware, Franklin Lakes, NJ, USA) as described previously (Librach *et al.* 1991) with minor modifications. Briefly, primary CT cells (2×10^5) and HTR-8/SVneo cells (1×10^5) were resuspended in the medium with or without IL8 (1 and 5 ng/ml), function-blocking anti-IL8 antibodies, or non-immune IgG (1 µg/ml). Cells were then seeded in the upper chamber of Matrigel-coated transwells. Insert membranes were previously coated on the upper side with 10 µl of growth factor-reduced Matrigel at 5 mg/ml by incubation for 30 min at 37 °C. The lower chambers were loaded with 500 µl of medium with or without IL8, anti-IL8, or non-immune IgG antibodies. Cells were incubated for 24 h at 37 °C in 5% CO₂. The chamber was disassembled, filters were washed twice in warm PBS, and cells on the upper surface were gently removed with a cotton swab. Cells were fixed in 4% paraformaldehyde with 0.05% Tween (v/v) for 30 min at RT. Filters were stained using primary anti-cytokeratin-18 for primary trophoblast by the same procedure as described above, and by Giemsa staining for HTR-8/SVneo cells. Cells on the underside of the filters and the pores occupied were counted in 40 randomly selected non-overlapping fields of the membranes under a light microscope (Reichert-Jung with Leica DC150 Digital Camera System). The mean of the controls was set to 100%, and the data are expressed as the percentage of the control values. Experiments were performed four times in duplicate for isolated trophoblast and six times in duplicate for HTR-8/SVneo cells. Data presented for HTR-8/SVneo were normalized to cell numbers (increased by IL8 as assessed by the MTT test). In the Matrigel invasion test, isolated CT differentiates to non-proliferative trophoblast.

Determination of viable cell number

The MTT test was used as an indicator of cell number (Hanisch *et al.* 1993). HTR-8/SVneo cells were cultured in 96-well plates as described above. At the end of the incubation interval, 100 µl of MTT (2.4 mmol/l) in 10% FCS/PBS (v/v) were added to each well. After incubation for 2 h at 37 °C, medium was replaced by 1-propanol (100 µl/well), and the plates were vigorously shaken to ensure complete solubilization of the blue formazan. Absorbance was measured at 570 nm using a microplate reader (LKB), and cell numbers were determined using a standard curve obtained with 5×10^3 , 1×10^4 , 2×10^4 , 4×10^4 , 6×10^4 , or 8×10^4 cells/well. The experiment was repeated five times, $n=12$.

Gelatin zymography

HTR-8/SVneo cells were incubated as described above. Gelatinase activities in serum-free conditioned media of HTR-8/SVneo cells were determined using SDS-polyacrylamide gel zymography as described previously (Lash *et al.* 2005). Samples were electrophoresed on 11% SDS-polyacrylamide gels containing 1 mg/ml of gelatin under non-reducing conditions. All samples for comparison were run on the same gel, with 25 µg of protein loaded per lane. Following electrophoresis, gels were washed twice for 15 min in 2.5% Triton X-100 (v/v) to remove SDS, and were then rinsed in dH₂O. After overnight incubation in reaction buffer (50 mmol/l Tris-HCl, pH 7, containing 5 mmol/l CaCl₂) at 37 °C, gels were stained with Coomassie brilliant blue R-250 for 30 min at RT and were destained in 30% methanol and 10% glacial acetic acid (v/v). Proteinase activity was observed as a clear band of digested gelatin. Gelatinase levels were semi-quantitated by densitometric analysis of zymograms using the ImageMaster TotalLab v2.01 programme (Amersham Biosciences). All zymography experiments were repeated at least three times in duplicate.

SDS-PAGE and immunoblotting

SDS-PAGE was performed on 10% polyacrylamide gel and 4% stacking gel under reducing conditions. All samples (HTR-8/SVneo cell lysates) were prepared by boiling in 0.125 M Tris-HCl buffer containing 4% SDS (w/v), 20% glycerol (v/v), 0.1% bromophenol blue, and 10% 2-mercaptoethanol (v/v) for 5 min, and 80 µg of protein were loaded per lane. SDS-PAGE standards (Bio-Rad) were used as molecular weight markers. Proteins separated by electrophoresis were transferred onto nitrocellulose membranes. Transfer was performed at constant power (1.2 mA/cm²) for 1.5 h, and was confirmed by Ponceau S staining of the membranes. Non-specific binding was blocked with 1% casein in PBS (w/v) overnight at 4 °C. After blocking, membranes with immobilized antigens were incubated with monoclonal anti-integrin α_1 , α_5 , or β_1 antibody overnight at 4 °C with constant shaking. After five washes for 5 min each, membranes were incubated with the anti-mouse IgG biotinylated secondary antibody for 30 min. After intensive washing, membranes were incubated with ABC for 30 min. Bound conjugates were visualized using DAB/Ni as the chromogen. Non-specific binding was estimated by omitting

the specific antibody. Staining for actin was used as the loading control. Membranes ($n=3$) were examined on a HP Scanjet G3110 scanner, and were analyzed by the ImageMaster TotalLab v2.01 programme (Amersham Biosciences).

Statistical analysis

The data were analyzed statistically with the Statistical Software Program, version 5.0 (Primer of Biostatistic, McGraw-Hill Companies, Inc., New York, NY, USA) using the non-parametric Mann–Whitney rank sum test and Student's t -test as appropriate. Values were considered significantly different when $P<0.05$.

Declaration of interest

The authors declare that there is no conflict of interest that could affect the impartiality of the research reported.

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