Stromal cell-derived factor-1 (SDF-1) signalling regulates human placental trophoblast cell survival

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Stromal cell-derived factor-1 (SDF-1 or CXCL12) is the physiologic ligand for the chemokine receptor CXCR4. CXCR4-mediated signalling regulates cell migration and apoptosis in certain haematopoietic and neuronal cells. Using gene profiling, we determined that CXCR4 is the only chemokine receptor for which mRNA expression is regulated during trophoblast differentiation in vitro. Based on the known effects of CXCR4 ligation, we hypothesized that CXCR4 activation may regulate placental trophoblast cell survival (i.e. protection from apoptosis), an important mechanism for the establishment and maintenance of the uteroplacental barrier. Human cytotrophoblasts (CTBs) were cultured in defined media and treated with graded doses of SDF-1 (10–100 ng/ml) or with an anti-CXCR4 neutralizing antibody. Exposure to anti-CXCR4 antibody reduced CTB cell numbers by 25–40%. Treatment with SDF-1 decreased the proportions of apoptotic terminal deoxynucleotidyl transferase-mediated dUTP-FITC nick-end labelling (+) cells (apoptotic index [AI] of 2.79 ± 0.61% [control] versus 1.88 ± 0.56% [SDF-1]; P < 0.05) and caspase-activated cells (AI of 7.95 ± 2.49% [control] versus 3.81 ± 1.49% [SDF-1]; P < 0.05). We determined that SDF-1 also activated the triple MAP Kinase isoforms ERK1/2 and p38 in trophoblasts. Immunocytochemistry confirmed SDF-1-induced nuclear translocation of phosphorylated ERK1/2. Blocking of ERK1/2 signalling with the specific inhibitor PD98059 reversed SDF-1-mediated inhibition of apoptosis (AI of 1.65 ± 0.34 [SDF-1] versus 3.50 ± 0.5 [SDF-1 + PD98059]; P < 0.05), suggesting that SDF-1 acts through this pathway as a trophoblast survival factor. These results indicate that SDF-1/CXCR4 signalling stimulates anti-apoptotic pathways in cultured trophoblasts. This chemotactic ligand/receptor system may promote trophoblast survival during pregnancy. Alterations in SDF-1 and/or CXCR4 expression or function may be associated with specific pregnancy disorders.

Key words: apoptosis/chemokines/placenta/trophoblast

Introduction

The chemokine receptor, CXCR4, a member of the heptahelical G protein-coupled receptor superfamily, was first identified as an orphan receptor (HUMSTR, LESTR or Fusin) (Loetscher et al., 1994). The CXCR4 ligand is stromal cell-derived factor-1 (SDF-1, systematic name CXCL12) (Zlotnik et al., 2000). SDF-1 was originally isolated from murine bone marrow stromal cells and characterized as a pre-B cell stimulatory factor (Nagasawa et al., 1994). Although most chemokines are pleotropic and activate multiple receptors, SDF-1 actions are mediated exclusively via binding to CXCR4, which also serves as a co-receptor for the X4 HIV-1 envelope (env) protein (Bleul et al., 1996a). SDF-1 and CXCR4 show a high degree of sequence conservation (>90% identity across diverse species) (Crump et al., 1997; Moepps et al., 1997) and appear to play a fundamental role in development (Nagasawa et al., 1996; Tachibana et al., 1998; Zou et al., 1998).

SDF-1 is a potent chemotactic factor for T cells (Kantele et al., 2000), monocytes (Bleul et al., 1996b), B cells (D’Apuzzo et al., 1997; Bleul et al., 1998; Corcione et al., 2000), dendritic cells (Sozzani et al., 1997), mast cells (Lin et al., 2000), eosinophils (Nagase et al., 2001) and CD34+ haematopoietic progenitors (Aiuti et al., 1997; Mohle et al., 1998; Wang et al., 1998). SDF-1 regulates homing of haematopoietic stem cells to the bone marrow (Kawabata et al., 1999; Naiyer et al., 1999), megakaryocyte transepithelial migration (Hamada et al., 1998; Wang et al., 1998), platelet aggregation (Abi-Younes et al., 2000), and differentiation of early B cell, megakaryocytic and erythroid lineages (Nagasawa et al., 1996; Ma et al., 1998; Kowalska et al., 1999; Majka et al., 2000). It also induces endothelial (Feil et al., 1998) and neuronal migration (Bajetto et al., 1999).

Apoptosis, a form of cell death distinct from necrosis, is characterized by cell shrinkage, nuclear chromatin condensation, internucleosomal DNA fragmentation, plasma membrane blebbing and formation of apoptotic bodies. SDF-1 enhances cell survival by inhibiting apoptosis in CD34+ cells (Lataillade et al., 2000), CD4+ cells (Suzuki et al., 2001), myeloid precursor cells (Broxmeyer et al., 2003a) and embryonic retinal ganglionic cells (Chalasani et al., 2003). CXCR4 neutralization by monoclonal antibody has been reported to enhance apoptosis in vitro in non-Hodgkins lymphoma cells (Bertolino et al., 2002).

Apoptosis occurs at specific times and sites during normal human placental development (Smith et al., 1997; Mayhew et al., 1999; SDF-1 is a potent chemotactic factor for T cells (Kantele et al., 2000), monocytes (Bleul et al., 1996b), B cells (D’Apuzzo et al., 1997; Bleul et al., 1998; Corcione et al., 2000), dendritic cells (Sozzani et al., 1997), mast cells (Lin et al., 2000), eosinophils (Nagase et al., 2001) and CD34+ haematopoietic progenitors (Aiuti et al., 1997; Mohle et al., 1998; Wang et al., 1998). SDF-1 regulates homing of haematopoietic stem cells to the bone marrow (Kawabata et al., 1999; Naiyer et al., 1999), megakaryocyte transepithelial migration (Hamada et al., 1998; Wang et al., 1998), platelet aggregation (Abi-Younes et al., 2000), and differentiation of early B cell, megakaryocytic and erythroid lineages (Nagasawa et al., 1996; Ma et al., 1998; Kowalska et al., 1999; Majka et al., 2000). It also induces endothelial (Feil et al., 1998) and neuronal migration (Bajetto et al., 1999).

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Burton et al., 2003). Regulated activation or suppression of the apoptotic cascade appears to be required for the establishment of normal uteroplacental interactions. Conversely, disturbances in programmed cell death in placenta have been associated with abnormal pregnancy outcomes (Smith et al., 1997; Leung et al., 2001; Neale et al., 2003).

Using a gene profiling strategy, we determined that CXCR4 expression is down-regulated during trophoblast cell differentiation. Our findings indicate that SDF-1/CXCR4 signalling suppresses apoptosis and enhances trophoblast cell survival via an MAPK effector pathway.

Materials and methods

Primary culture of human trophoblasts
Placental cytotrophoblasts (CTBs) were isolated as previously described (Rubin et al., 1993). Briefly, term human placentas were obtained immediately after uncomplicated vaginal or Caesarean deliveries. Consent was obtained for the use of discarded tissue for research purposes using procedures approved by the IRB of Women & Infants’ Hospital of Rhode Island. Villous tissue was minced and digested twice with 0.125% trypsin and 0.02% deoxyribonuclease I (Sigma, St. Louis, MO, USA) in Hanks balanced salt solution containing 0.8 mM MgSO4 and 25 mM HEPES (pH 7.4). CTBs were isolated by centrifugation through 5–70% Percoll (Amer- sham Pharmacia Biotech, Piscataway, NJ, USA) step gradients at 2100 g for 20 min. The CTB layer was collected, washed and plated on fibro- nectin-coated dishes at a density of 1.0–1.5 £ 10^6 cells/dish in Dulbecco’s modified Eagle’s medium (DMEM) (Life Technologies, Rockville, MD, USA) containing 25 mM glucose, 20% fetal bovine serum, 100 mU/ml penicil- lin G and 100 mU/ml streptomycin sulphate. Cells were cultured at 37°C in 95% air/5% CO2. The presence of non-trophoblastic cells was determined in control cultures using mouse monoclonal anti-vimentin antibody (Sigma) and assessment for fibroblast morphology. CTB purities ranged from 90% to 97%. Under these experimental conditions, CTBs differentiate in culture and express time-dependent accumulation of mRNAs for chorionic gonadotropin-α (hCGα), hCGβ, placental lactogen (hPL) and progesterone (Rubin et al., 1993).

Differential display
Differential display RT–PCR (Liang and Pardee, 1998) was performed using a GenHunter RNAImage Kit (GenHunter Corp., Nashville, TN, USA) with minor modifications. Trophoblast total cellular RNA was isolated as described below and 25 µg aliquots were digested with 10 U of RNase-free DNase (Roche Applied Science, Indianapolis, IA, USA) in 100 µl buffer (50 mM KCl, 100 mM Tris–HCl, pH 8.0; 1.5 mM MgCl2, 10 U placental RNase inhibitor (Promega, Madison, WI, USA)) at 37°C for 45 min. RNA was re-extracted, precipitated in cold ethanol and resuspended in water at 1 mg/ml. RNA samples (0.2 mg) were reverse transcribed in triplicate tubes containing 20 pmol of H-T11N-anchored oligo-dT primers and H-AP arbitrary 13mers (GenHunter) in a final volume of 20 µl of PCR mix containing 10 mM dithiothreitol and 20 µM of each dNTP at 65°C for 5 min to denature mRNA secondary structure. After primer annealing at 37°C for 10 min, 10/µl of SuperScript II reverse transcriptase (Life Technologies) was added for 1 h followed by a second cycle of RNA template denaturation, cooling and reverse transcription.

PCR amplification was performed in a PTC-100 thermal cycler (MJ Research, Waltham, MA, USA) using 4 µl of the RT mix in 20 µl of PCR buffer containing 2 µM dNTPs, 20 pmol of H-T11N 3’-primers, 4 pmol of the arbitrary sequence 5’-primers, 10 µCi [a-32P]dATP (Amersham) and 0.2 U AmpliTaq DNA polymerase (PerkinElmer, Boston, MA, USA). The PCR programme was 94°C x 1 min (denaturation), 40°C x 2 min (annealing), 72°C x 30 s (extension) x 40 cycles followed by 72°C x 10 min (extension). 3’-End cDNAs (100–500bp) were visualized using 6% polyacrylamide/8 M urea gel electrophoresis and autoradiography. Band autoradiographic intensities (control versus experimental) were compared side-by-side in triplicate samples. Differentially displayed bands were eluted from the gel and the amplified PCR product (20 ng) was directly ligated into pCRII by TA cloning (Invitrogen Corp., Carlsbad, CA, USA) and transformed with InvA’ E. coli (Invitrogen) prior to sequencing.

RNA extraction and Northern blot analysis
Total cellular RNA was isolated as previously described (Sanchez-Esteban et al., 1998). RNA samples (20 µg/lane) were electrophoresed in 1.4% agar- oose gels containing 2.2 M formaldehyde and transferred by capillary blotting to nylon membranes (GeneScreen; Dupont NEN, Boston, MA, USA). For Northern blot hybridization, linearized cloned human CXCR4 (see Results) and hCGs (Sarkar et al., 2001) templates were used to synthesize [a-32P]UTP-labelled antisense cRNA probes by in vitro transcription (Promega). All blots were stripped and rehybridized with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) riboprobe (Sarkar et al., 2001) to normalize for loading and transfer discrepancies.

Ribonuclease protection assay (RPA)
Multiprobe RPA (RiboQuant, PharMingen, San Diego, CA, USA) were performed as per manufacturer’s protocol. [a-32P]UTP-labelled antisense CRNA probes were synthesized from human cytokine receptor multiprobe template sets (hCR-5 and hCR-6, PharMingen) by in vitro transcription. RNA (20 µg/tube) was mixed with 108 cpm of probe, precipitated in ethanol, resuspended in 20 µl of hybridization buffer (80% deionized formamide; 100 mM sodium citrate, pH 6.4; 300 mM NaAc, pH 6.4; 1 mM EDTA), denatured and hybridized overnight at 56°C. Unhybridized single-stranded RNA was digested by addition of RNase A + T1 at 37°C for 30 min. RNAses were inactivated in the same tubes and RNA was precipitated in cold ethanol. Protected fragments (RNA:RNA duplexes) were resolved on 5% polyacrylam- ide/8 M urea gels. For each experiment, control lanes contained probes hybridized to sheared yeast tRNA. Gels were mounted on filter paper and exposed to X-ray film with intensifying screens. Differences in band signal intensity were adjusted to intensities of the constitutively expressed L32 and GAPDH RPA bands.

Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-FITC nick-end labelling (TUNEL) assay
Detection and quantification of apoptotic trophoblast cells was performed using a fluorescein-labelled apoptosis detection kit (DeadEnd Fluorometric TUNEL System, Promega). CTBs cultured in 6-well dishes were pre-incu- bated overnight in DMEM containing 1% FBS+ (insulin, transferrin, se- leneous acid) + linoleic acid (BD Biosciences, Bedford, MA, USA) and treated with 50 ng/ml SDF-1 (R&D Systems, Minneapolis, MN, USA) or 10 µg/ml mouse anti-human CXCR4 antibody (Biosource International, Camarillo, CA, USA) and incubated at 37°C overnight. Cells were washed with phosphate-buffered saline (PBS), fixed with freshly prepared 4% paraformalde- hyde in PBS at 4°C for 25 min, re-washed and permeabilized with 0.2% Triton X-100. Samples were incubated in equilibration buffer containing flu- orescein-12-dUTP nucleotide mix and TdT enzyme in a humidified chamber at 37°C for 60 min, washed in PBS and analysed by fluorescence microscopy. At least 500 cells in a minimum of eight randomly chosen high-power fields were photographed and analysed using NIH Image V1.61, by an operator masked to the treatment conditions. Apoptotic index (AI), defined as the per- centage of TUNEL-stained nuclei divided by the total number of nuclei (stained with propidium iodide), was calculated for each condition.

Total caspase activity
Caspase activation was quantified by cytofluorochrome using a carboxy- fluorescein-labelled caspase substrate (CaspaTag, Intergen, Purchase, NY, USA). CTBs were treated with SDF-1 or anti-CXCR4 antibody at 37°C for 3 h followed by incubation with FAM-VAD-FMK caspase substrate at 37°C for 1 h. Hoechst 33258 was added for nuclear labelling. Samples were fixed in 1% paraformaldehyde and analysed as described above.

Western blotting
CTBs in 60 mm culture dishes (1.5 x 105 cells/dish) were incubated in DMEM with 1% ITS+ at 37°C for 3 h and treated with SDF-1 (50 ng/ml) for
the indicated times. Trophoblast cultures were lysed and protein extracts analysed with a 1:500 dilution of anti-CXCR4 antibody (Calbiochem, San Diego, CA, USA). Subcellular fractionations were performed as described elsewhere (Boylan and Gruppuso, 2002). For MAPK activity, cells were lysed with 200 μl of MAPK lysis buffer (25 mM β-glycerophosphate, 1 mM EDTA, 100 mM NaCl, 10 mM MgCl2, 500 mM NaF, 0.2 mM sodium orthovanadate, 1% Triton X-100, protease inhibitors). Lysates were pelleted and supernatants were stored at −80°C. Protein content was determined by BCA assay (Pierce Chemical, Rockford, IL, USA). Protein samples (30 μg/lane) were separated by 10% sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes (Bio-Rad, Hercules, CA, USA). Membranes were incubated at 37°C in blocking solution (Tris-buffered saline-Tween 20 [TBS-T] with 5% non-fat dry milk) followed by incubation with specific anti-phospho-MAPK antibodies (1:1000; Cell Signaling, Beverly, MA, USA) at room temperature for 1 h, and then with donkey anti-rabbit horseradish peroxidase-labelled secondary antibody (1:2000; Jackson Immunochemicals, West Grove, PA, USA). Phosphorylated MAPKs were detected by enhanced chemiluminescence (ECL, Amersham). Blots were stripped (2% SDS; 62.5 mM Tris–HCl, pH 6.7; 100 mM 2-mercaptoethanol) and hybridized in a similar fashion as total MAPK activity.

**MAP Kinase inhibition**

MAPK pathways were blocked using specific inhibitors for ERK1/2 (50 μM PD98059; Cell Signaling), p38 (10 μM SB203580; Calbiochem-Novabiochem, San Diego, CA, USA) and JNK (20 μM SP600125; Bionol, Plymouth Meeting, PA, USA). CTBs cultured in 6-well dishes were pre-incubated overnight in DMEM containing 1% ITS-. Cells were then incubated with 50 ng/ml SDF-1 or MAPK inhibitor or SDF-1 + MAPK inhibitor at 37°C overnight. Cells treated with MAPK inhibitor or SDF-1 + MAPK inhibitor were pre-treated with the specific MAPK inhibitor at 37°C for 1 h, while the control and SDF-1-treated cells were pre-treated with vehicle dimethylsulphoxide only. TUNEL assay results are reported as AI.

**Statistical analysis**

Data for TUNEL and total caspase activity were analysed by Student’s t-test. P < 0.05 was considered to be significant. Results are displayed as mean ± SE of the mean. Statistical calculations were performed using the Statistica version 4 software.

**Results**

**CXCR4 expression is regulated in differentiating trophoblasts**

The peptide hormone calcitonin promotes trophoblast maturation assayed by glycoprotein production (Rebut-Bonneton et al., 1992) and syncytium formation (Rubin, unpublished results). Therefore, calcitonin was used as a physiological inducer of CTB differentiation for differential display experiments. CTBs maintained in a defined medium (DMEM/1% ITS-) were incubated in 100 nM calcitonin for 6 h. As described in Materials and methods, triplicate side-by-side comparisons of calcitonin-treated and untreated RT–PCR samples revealed several up- and down-regulated partial cDNAs. A human CXCR4 3′-cDNA was sequenced among seven calcitonin-suppressible cDNAs detected.

The CXCR4 3′-end cDNA was extended by 5′-RACE (5′-rapid amplification of cDNA ends) and used to synthesize an antisense cRNA probe. Calcitonin-inducible down-regulation of CXCR4 expression was confirmed by Northern blotting (Figure 1). In order to determine whether CXCR4 is similarly regulated during spontaneous CTB differentiation over time in vitro, trophoblast RNA was isolated daily for 4 days (CTB monolayers cultured in the presence of serum, biochemically differentiated and fused into syncyta by day 3). Trophoblast expression of chemokine receptors was then assayed by multiprobe RPA. Figure 2 indicates that differentiating CTBs express several chemokine receptors. CXCR4, uniquely among these trophoblast chemokine receptor mRNAs, decreased as term trophoblasts differentiated in culture from day 1 to day 4.

In parallel trophoblast samples, total cellular protein was isolated and assayed for CXCR4 by Western blot. We found that CXCR4 protein levels increased significantly several hours after the detected peak in CXCR4 mRNA (Figure 3A) and then declined in association with further cell differentiation. This developmental pattern is also seen for several other CTB membrane proteins, e.g. E-cadherin is down-regulated co-incident with cell fusion (Coutifaris et al., 1991). Trophoblast protein samples were subjected to subcellular fractionation in order to isolate membrane-associated proteins. Western blotting of these protein fractions revealed that trophoblast CXCR4 was localized largely to the membrane-bound fraction (Figure 3B). This finding is consistent with CXCR4 function as a heptahelical transmembrane receptor. Trophoblast CXCR4 appears to be a functional receptor. We determined that SDF-1 treatment of the cells decreased expression of a trophoblast gene, hCGx (Figure 4). Although low levels of trophoblast SDF-1 mRNA could be detected by RT–PCR, no SDF-1 protein was measurable in culture supernatants using a highly sensitive ELISA (R&D Systems, Minneapolis, MN; data not shown). These findings prompted an investigation into whether SDF-1/CXCR4 signalling might be an important, hitherto unexpected, effector pathway in placental biology.

**Anti-apoptotic effects of trophoblast SDF-1/CXCR4 signalling**

Subsequent studies were performed on day 2 of cell culture, when surface expression of CXCR4 was maximal, a finding similar to a previous observation (Athanassakis et al., 2000). The effect of SDF-1/CXCR4 signalling on cell survival was determined using complementary assays of apoptosis. Figure 5A shows representative fields from TUNEL assay fluorescence photomicrographs of cultured trophoblasts. TUNEL-labelled (fluorescein-positive) nuclei also exhibited characteristic nuclear condensation and blebbing. Overnight treatment of cells with SDF-1 (50 ng/ml) significantly reduced apoptosis (Figure 5B). The AI decreased from 2.79 ± 0.61% (mean ± SE) in control cells to 1.88 ± 0.56% in SDF-1 treated CTBs (P < 0.05). In contrast, addition of an anti-CXCR4 neutralizing antibody increased apoptosis (6.0 ± 0.87%) significantly compared to both controls and to SDF-1-treated cells (P < 0.05) (Figure 5B).

Analysis of total caspase activation revealed a similar anti-apoptotic effect of SDF-1 in differentiating CTBs. In this assay, caspase activation cleaves the FAM-VAD-FMK substrate to a fluorescein-labeled fragment. Figure 6A shows representative fields in which Hoechst 33258-stained nuclei are pseudo-coloured red for contrast.
Treatment with SDF-1 significantly decreased the AI (3.81 ± 1.49%) from control values (7.95 ± 2.49%; \( P \), 0.05). These findings indicate that SDF-1 inhibits caspase-dependent apoptosis. Conversely, anti-CXCR4 neutralizing antibody-treated cells had a significantly higher AI (7.2 ± 1.2%) when compared to SDF-1-treated CTBs, although an increase in apoptosis was not seen when compared to the controls (Figure 6B).

**Trophoblast SDF-1/CXCR4 signalling activates MAP Kinases**

In order to identify intracellular signalling pathways required for SDF-1/CXCR4-mediated protection from apoptosis, we examined the effect of ligand-dependent receptor activation on trophoblast MAPK signalling. SDF-1 increased phosphorylation of ERK1/2 (Figure 7B). A similar, more robust activation was seen for p38 MAPK (Figure 7A). SDF-1 phosphorylation of MAPKs was rapid and biphasic. SDF-1-inducible ERK1/2 phosphorylation increased with time and peaked at 5 min. Increased p38 phosphorylation was seen at 2 min followed by a rapid return to basal levels. In contrast, we obtained variable results for SDF-1 effects on the MAPK isoform JNK. Modest increases in JNK phosphorylation were observed at 2 and 5 min in several, but not all, placental cultures tested (results not shown).

**SDF-1/CXCR4 inhibition of trophoblast apoptosis is ERK1/2 MAPK dependent**

In order to determine whether MAPK activation is required for the apparent anti-apoptotic effect of SDF-1, ERK1/2 phosphorylation...
was blocked using the MEK1 inhibitor, PD98059, and the apoptotic activity was measured by a TUNEL assay (Figure 8). AI for SDF-1-treated CTBs was 1.65 ± 0.83%, significantly lower (P < 0.05) than for cells pre-incubated with PD98059 and treated with both PD98059 and SDF-1 (3.5 ± 0.5%). PD98059 alone had no effect on trophoblast apoptosis (AI of 3.4 ± 0.37%). AI for controls was 3.15 ± 0.15%.

In contrast, inhibition of p38 with SB203580 had no effect on SDF-1-suppressible CTB apoptosis. The AI of SDF-1-treated CTBs (3.12 ± 0.76%) did not differ from that of CTBs treated with both SB203580 and SDF-1 (1.29 ± 0.39%). Inhibition of JNK with SP600125 similarly had no effect on SDF-1-suppressible apoptosis; AI for SDF-1 of 5.48 ± 1.92% versus AI for SDF-1 plus SP600125 of 3.75 ± 3.25%. This difference was not statistically significant.

### Discussion

The developing blastocyst attaches to the uterine lining via trophoblast implantation. Invasive trophoblasts breach the uterine epithelium, penetrate deeply into the uterine endometrium and cluster in the perivascular endometrial stroma (Pijnenborg, 1990). As a consequence, uterine blood flow is modified and haemochorial placenta- tion is established. Unlike tumour invasion, trophoblast invasion is a precisely regulated process (Bischof et al., 2000) with a fine balance between pro- and anti-apoptotic factors. Dysregulation of trophoblast attachment, migration or invasiveness may lead to several common complications of pregnancy, including faulty implantation, early pregnancy loss, pre-eclampsia and intrauterine growth restriction.

Chemokines and their receptors regulate numerous types of cell migration. Although currently little information is available on the role played by chemokines and chemokine receptors in normal placental development, the presence of several chemokine receptors on differentiating trophoblasts suggests they are likely important (Drake et al., 2004). Murine (Athanassakis et al., 2001) and human (Moussa et al., 1999; Mognetti et al., 2000) trophoblast-derived cells express CXCR4. CXCR4 expression has been demonstrated both in early and relatively undifferentiated CTBs (Athanassakis et al., 2000) and in differentiated syncytiotrophoblasts by in vitro primary cultures (Douglas et al., 2001). Additionally, SDF-1 staining of trophoblast and amnion has been reported (Coulomb-L’Hermine et al., 2000).

Another line of evidence implying a role of this pathway in uteroplacental development is that CXCR4 and SDF-1 knockout mouse embryos have identical embryonic lethal phenotypes. These fetal mice show ventricular septal defects, vascular malformations, abnormal cerebellar neuronal migration, B cell deficiency and impaired haematopoietic colonization of the bone marrow (Nagasawa et al., 1996; Ma et al., 1998; Tachibana et al., 1998; Zou et al., 1998). None of these described abnormalities, however, adequately explains the nearly uniform late gestational lethality of SDF-1 and CXCR4 null mice. Based on our in vitro findings in human cells, we speculate that absence of SDF-1 or CXCR4 may result in significant uteroplacental pathology.

The current study demonstrates that CXCR4/SDF-1 signalling plays an important role in trophoblast survival. Consistent with the concept that CXCR4 is important in placental development, uniquely among chemokine receptors surveyed, we found CXCR4
gene expression is regulated during trophoblast differentiation in vitro. The CXCR4 is functional as SDF-1 increases a trophoblast gene product, hCGα, activates ERK1/2 and suppresses apoptosis. Subcellular fractionation showed that membrane-bound trophoblast CXCR4 accumulation follows maximal expression of CXCR4 mRNA. CXCR4 is known to traffic rapidly between the cell surface and intracellular compartments (Maldonado-Estrada et al., 2003). In our studies, the differentiation- and time-dependent decline in CXCR4 apparently results from inhibition of gene expression rather than intracellular redistribution of cell surface receptors.

In our experiments, apoptosis in cultured term trophoblasts measured by TUNEL was comparable to previous reports (Levy et al., 2000; Crocker et al., 2001). Moreover, TUNEL-positive cells exhibited morphologic features characteristic of apoptosis. We determined that SDF-1 reduced trophoblast apoptosis significantly in this experimental system. The measurement of total caspase activity revealed a similar anti-apoptotic effect of SDF-1. Caspases, a family of cytoplasmic aspartate-specific cysteine proteases, are critical regulators of cell death initiation and execution phases (Green, 1998). The inhibition of caspase activity indicates that the effect of SDF-1 is, at least in part, via inhibition of caspase-dependent apoptotic pathways.

SDF-1/CXCR4 interactions trigger several intracellular signals in different cell types including increases in cytosolic calcium ion flux,
ERK1/2 phosphorylation, activation of phosphatidylinositol 3-kinase and Akt, tyrosine phosphorylation of focal adhesion complex components such as Pyk-2 and Crk and increases in NF-κB activity (Vlahakis et al., 2002). In trophoblasts, we have shown that SDF-1 activates two principal MAPK isofoms: ERK1/2 and p38. The activation of ERK1/2 was required specifically for the anti-apoptotic effect of SDF-1. These are the first data directly implicating this pathway in SDF-1-mediated cell survival and they differ from results obtained in haematopoietic cells. In CD34+ cells, SDF-1 has been shown to promote cell survival via pathways other than ERK1/2–MEK activation (Kijowski et al., 2001; Lataillade et al., 2002; Vlahakis et al., 2002). Nevertheless, apoptosis is a complex process regulated by different extracellular signals and intracellular effector pathways in different cell types. These conflicting data regarding intracellular mechanisms of SDF-1-mediated protection from apoptosis, as well as previous findings that SDF-1/CXCR4 signalling increases neuronal cell death (Hesselgesser et al., 2002), highlight the complexity of SDF-1-mediated signalling depending on the cell type and experimental conditions.

We conclude that CXCR4 is a uniquely regulated trophoblast chemokine receptor. In this report, we focused on the role of SDF-1/CXCR4 signalling on near-term and term trophoblast cell survival. Our preliminary observations indicate a similar effect of SDF-1/CXCR4 signalling on both apoptosis and primary necrosis. Placenta 24, 297–305.

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