

REGULAR ARTICLE

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Differentiation-dependent expression of gelatinase B/matrix metalloproteinase-9 in trophoblast cells

Received: 5 June 1998 / Accepted: 26 August 1998

Abstract The purpose of this study was to evaluate the Rcho-1 trophoblast culture system as a model for studying trophoblast invasion and to examine stage-specific expression of enzyme(s) potentially participating in rat trophoblast giant cell invasive behavior. The invasive behavior of the differentiating Rcho-1 trophoblast cells was demonstrated using Matrigel invasion chambers. Gelatin zymography and Western blot analysis of conditioned medium from differentiating Rcho-1 trophoblast cell cultures and rat ectoplacental cone outgrowths revealed a differentiation-dependent increase in gelatinase B/matrix metalloproteinase (MMP-9). Northern blot and reverse transcriptase polymerase chain reaction (RT-PCR) analyses of Rcho-1 trophoblast or ectoplacental cone cells also showed increasing expression of MMP-9 accompanying cell differentia-

tion. Rcho-1 trophoblast cells stably transfected with MMP-9 promoter/luciferase reporter constructs exhibited a differentiation-dependent increase in MMP-9 promoter activation. In conclusion, trophoblast giant cell differentiation is characterized by transcriptional activation of the MMP-9 gene and appearance of the invasive phenotype.

Key words Trophoblast giant cell · Placenta · Gelatinase B/MMP-9/92-kDa gelatinase · Ectoplacental cone outgrowths · Invasion, trophoblast · Rat (Sprague Dawley)

This work was supported by grants from the National Institute of Child Health and Human Development (HD 20676, HD 33994). T.J.P. was supported in part by the W.S. Sutton Award and a fellowship from the Kansas Health Foundation.

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Introduction

Hemochorial placentation (e.g., rodents and primates) is characterized by trophoblast-cell-orchestrated modification of the maternal uterine environment into a structure promoting nutrient flow to the developing embryo/fetus (Aplin 1991; Enders and Welsh 1993). In order to accomplish this important task, trophoblast cells acquire the capacity to produce hormones/cytokines that redirect maternal resources to the uterus (Soares et al. 1996) and proteases that effectively place the developing embryo in direct proximity to a rich nutritional supply (Salamonsen 1994; Hulbooy et al. 1997; Rinkenberger et al. 1997). Among the proteases produced by developing trophoblast cells are members of a group of zinc-dependent endopeptidases (Salamonsen 1994; Hulbooy et al. 1997; Rinkenberger et al. 1997). These enzymes are capable of degrading extracellular matrix components and are referred to as matrix metalloproteinases (MMPs; Nagase et al. 1992; Birkedal-Hansen et al. 1993).

In the rat and mouse, trophoblast cells located at the maternal interface achieve a unique phenotype and are referred to as trophoblast giant cells. These cells arise from precursors by a process referred to as endoreduplication and possess endocrine activities and the potential for invasion (Enders and Welsh 1993; Gardner and Beddington

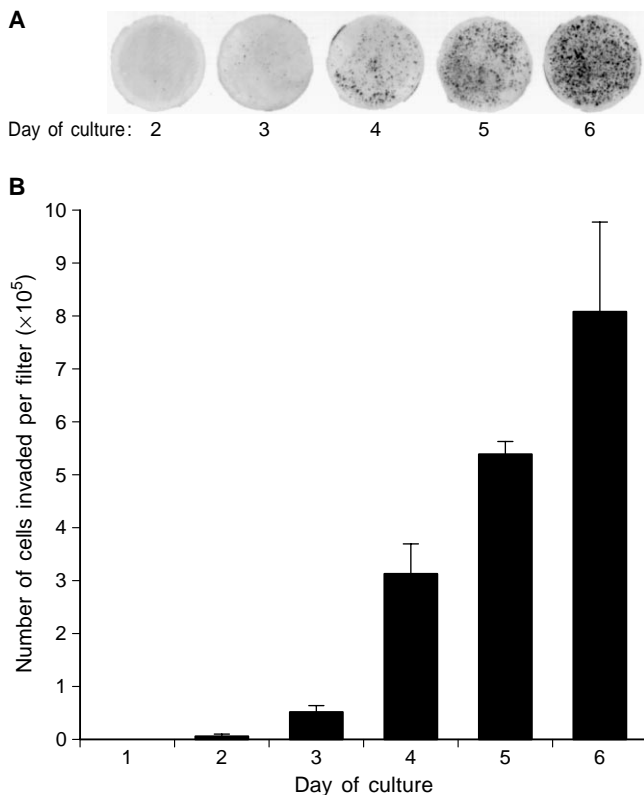


Fig. 1A, B Rcho-1 trophoblast matrigel invasion assay. Rcho-1 trophoblast cells were plated on matrigel invasion chambers and the lower sides of porous filter supports were stained to allow manual counting of invaded cells after various times of culture. **A** Representative photomicrograph of stained Rcho-1 trophoblast cells that invaded and attached to the underside of matrigel-coated filter supports after 2–6 days of culture. $\times 1$ **B** Graphical depiction of Rcho-1 trophoblast invasion after 1–6 days of culture. All experiments were performed in triplicate and repeated at least 3 times. Data are expressed as the mean \pm standard error of the mean

1988; Soares et al. 1995). Trophoblast giant cells express and secrete MMPs, including MMP-9, which is also referred to as gelatinase B (Behrendtsen et al. 1992; Harvey et al. 1995; Sharkey et al. 1996; Alexander et al. 1996; Das et al. 1997). MMP-9 is a 92-kDa protein that possesses proteolytic activity on gelatin (denatured type I collagen), collagens types IV, V, and VIII, and some noncollagenous proteins including casein and fibrinogen (Nagase et al. 1992; Birkedal-Hansen et al. 1993). Inhibition of MMP-9 activity with specific antibodies or inhibitors has directly implicated MMP-9 in the process of trophoblast invasion (Librach et al. 1991; Behrendtsen et al. 1992).

An *in vitro* culture model, the Rcho-1 trophoblast cell line, has been established that effectively reproduces many aspects of the differentiation of trophoblast giant cells (Faria and Soares 1991). Rcho-1 trophoblast cells were established from a transplantable rat choriocarcinoma (Faria and Soares 1991) and can be maintained in a proliferative state or induced to differentiate into trophoblast giant cells that morphologically and functionally resemble trophoblast giant cells developing *in situ* (Faria and Soares 1991; Hamlin et al. 1994; Yamamoto et al. 1994; Hamlin and Soares

1995). The proliferative Rcho-1 trophoblast cells share similarities with cells isolated from the ectoplacental cone (Verstuyf et al. 1992). Under *in vitro* conditions, when induced to differentiate, Rcho-1 trophoblast cells activate a trophoblast giant cell endocrine phenotype demonstrating the capacity to synthesize and secrete both steroid and peptide hormones (Faria and Soares 1991; Hamlin et al. 1994; Yamamoto et al. 1994). When transplanted beneath the kidney capsule, the Rcho-1 trophoblast cells exhibit both endocrine and invasive phenotypes (Faria et al. 1990b); however, it is unclear whether the invasive behavior is attributed to an activity related to the trophoblast giant-cell-differentiated phenotype or represents the transformed nature of the cells.

In this report, we examine the invasive phenotype of Rcho-1 trophoblast cells during various stages of their differentiation toward trophoblast giant cells. We show differentiation-dependent transcriptional activation of the MMP-9 gene and acquisition of the invasive phenotype in trophoblast giant cells that parallels the pattern of trophoblast cell differentiation from the ectoplacental cone.

Materials and methods

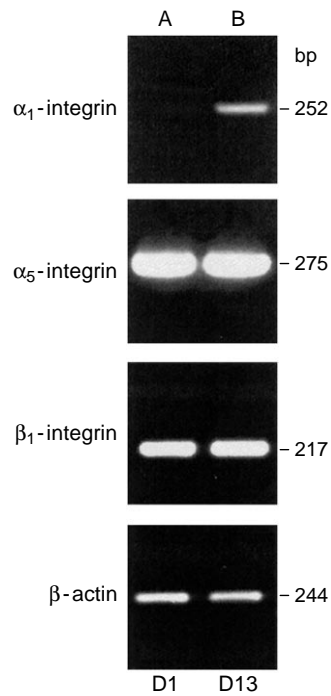
Reagents

Horse serum (HS) was obtained from JRH Scientific (Lenexa, KS). Reagents for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), protein assays, and prestained broad-range protein standards were purchased from Bio-Rad (Hercules, CA). Nitrocellulose and nylon membranes were acquired from Schleicher and Schuell (Keene, NH). X-Omat AR X-ray film was obtained from Eastman Kodak (Rochester, NY). DNA extraction kits were purchased from Qiagen (Chatsworth, CA). Prime-it random primed DNA-labeling kit and *Pfu* polymerase were purchased from Stratagene (La Jolla, CA). α -[32 P] deoxyadenosine triphosphate was purchased from DuPont-New England Nuclear (Boston, MA). All restriction enzymes and DNA ligases were obtained from New England Biolabs (Beverly, MA). BioCoat matrigel invasion chambers and uncoated polycarbonate filter inserts were purchased from Collaborative Biomedical Products (Bedford, MA). Diff-Quick differential stain was acquired from Baxter (McGaw Hill, IL). Lipofectamine, Opti-MEM, subcloning competent DH5 α cells, Superscript preamplification kits, *Taq* polymerase, and oligonucleotide primers were obtained from GibcoBRL (Gaithersburg, MD). Luciferase assay kits, pGL-2 basic vector, and an RSV promoter-luciferase reporter plasmid were acquired from Promega (Madison, WI). Antibodies to *cdc2* kinase were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Monoclonal antibodies for rat MMP-9 were obtained from Drs. Henning Birkedal-Hansen and Jeffrey A. Engler (University of Alabama, Birmingham, AL). ECL Western blot detection kits were purchased from Amersham Life Science (Arlington Heights, IL). A partial cDNA, 8p2A, for rat MMP-9 was obtained from Dr. Ruth Muschel (University of Pennsylvania, Philadelphia, PA). A cDNA for *cdc-2* kinase, the pSV₂Neo vector, and the human fibrosarcoma cell line, HT-1080, were acquired from American Type Tissue Collection (Rockville, MD, USA). Unless indicated otherwise all other reagents and chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Animals and tissue dissections

Holtzman rats were obtained from Harlan Sprague-Dawley Inc. (Indianapolis, IN). Rats were housed in an environmentally controlled fa-

Fig. 2 Integrins and invasive phenotype in Rcho-1 trophoblast cells. RT-PCR analysis for α 1-, α 5-, and β 1-integrin expression (upper panels) or β -actin (lower panel) in proliferative and differentiated Rcho-1 trophoblast cells. Lane 1 proliferative trophoblast cells (day 1 of culture); lane 2 differentiated trophoblast giant cells (day 13 of culture)



cility, with lights on from 0600 to 2000 hours, and allowed free access to food and water. Timed pregnancies were generated by cohabitation of female and male rats and daily examination of vaginal smears. The presence of a copulatory plug or sperm in the vaginal smear was designated day 0 of pregnancy. Rat ectoplacental cone tissues were dissected as previously described (Albieri and Bevilacqua 1996). Briefly, conceptuses were recovered on day 8 of pregnancy and maintained in sterile phosphate-buffered saline (PBS, pH 7.2) containing 10% fetal bovine serum (FBS). Embryos were isolated from the decidua and the ectoplacental cone (seen as a cone-shaped vascularized region) was separated from the embryo. Protocols for the care and use of animals were approved by the University of Kansas Animal Care and Use Committee.

Cell culture

Rcho-1 trophoblast cells were cultured as previously indicated (Faria and Soares 1991). Briefly, Rcho-1 trophoblast cells were maintained in a proliferative subconfluent state in NCTC-135 culture media supplemented with 10 mM hydroxyethylpiperazine ethanesulfonic acid (HEPES), 50 μ M β mercaptoethanol, 1 mM sodium pyruvate, 100 U/ml penicillin and 100 μ g/ml streptomycin, and 20% fetal bovine serum (FBS) in a 37°C incubator under 95% air/5% CO₂. After 3 days of culture in NCTC-135 culture medium supplemented with 20% FBS, cultures of Rcho-1 trophoblast cells were switched to NCTC-135 culture medium containing 10% HS serum to optimize differentiation (Hamlin et al. 1994; Peters et al. 1998). Trophoblast giant cell differentiation was verified by the morphological detection of trophoblast giant cells and/or the expression of placental lactogen-I (Peters et al. 1998). The HRP-1 trophoblast stem cell line, which was derived from labyrinthine trophoblast and exhibits directional transport properties (Soares et al. 1987; Shi et al. 1997), was maintained in RPMI-1640 medium containing 50 μ M β -mercaptoethanol, 1 mM sodium pyruvate, antibiotics, and 20% FBS and used as a control in the promoter analyses. HT1080 human fibrosarcoma cells were cultured in DMEM supplemented with antibiotics and 10% FBS and used as a source of MMP-2 and MMP-9 (Okada et al. 1992).

Invasion assay

Matrigel biocoat invasion chambers were rehydrated with NCTC-135 culture medium for 2 h at room temperature. Rcho-1 trophoblast cells were then seeded at 5×10^4 per 3 ml NCTC-135 containing 20% FBS and added separately to replicate chambers. Cells were then incubated at 37°C for 72 h under 95% humidity and 5% CO₂. Some chambers were washed with Hanks' balanced salt solution (HBSS) and changed to NCTC-135 with 10% HS to promote differentiation. Chambers were removed at the indicated times, matrix and cells on the upper surface of the chambers were scraped, and the membrane was fixed and stained with Diff-Quick. Chamber membranes were then excised and placed on slides, overlaid in immersion oil, and cells that invaded and attached to the undersurface of the chamber were manually counted using a microscope ocular grid. Ten fields per membrane were chosen at random for analysis and the total numbers of invaded cells per chamber were extrapolated by converting area of ocular to total area of chamber membrane. Each experiment was performed three separate times in triplicate.

Ectoplacental cone explant cultures

Ten to 14 ectoplacental cone explants were plated per well (96-well culture plate) in NCTC-135 culture medium supplemented with 20% FBS. Trophoblast giant cells began to grow out from the explants approximately 48 h following initial plating. Culture medium was changed every other day. On day 5 of culture, outgrowths were washed twice with HBSS and culture medium was changed to NCTC-135 containing 1% HS. After an additional 24 h, culture medium was harvested, clarified by centrifugation, and stored at -80°C until analyzed. Ectoplacental cone outgrowths were also harvested on day 3 or day 6 of culture for RNA analysis.

RT-PCR analysis

Total RNA was isolated from either Rcho-1 trophoblast cells or ectoplacental cone outgrowths according to the method of Chomczynski and Sacchi using TRIzol (Chomczynski and Sacchi 1987). RNA was reverse transcribed as previously reported (Rasmussen et al. 1997). Rat MMP-9-specific primers were generated to regions within exons 9 and 12 (Okada et al. 1995) and resulted in the amplification of a 443-bp product. Polymerase chain reaction (PCR) was performed with an upstream primer, 5' TTCGACTCCAGTAGACAATCC 3' (sense, 1533-1553; located in exon 9) and a downstream primer, 5' CAGAGAACCTCGTTATCCAAGCG 3' (antisense, 1954-1975; located in exon 12). The reverse transcriptase (RT)-PCR protocol included a hotstart series of 5 cycles, preceding 35 cycles of PCR with the denaturing temperature at 94°C (1 min), annealing temperature at 65°C (1 min), and extension temperature at 72°C (1 min). Primers and procedures for the RT-PCR analysis of α 1-, α 5-, and β 1-integrins were as previously described (Sutherland et al. 1993). RT-PCR analysis of β -actin was used to control for RNA integrity and accuracy of loading (Orwig et al. 1997).

Substrate gel electrophoresis (zymography)

The presence of metalloproteases in medium conditioned by the Rcho-1 trophoblast cell and ectoplacental cone outgrowths was determined by zymography as previously reported (Hibbs et al. 1985). Conditioned medium was mixed with SDS-PAGE nonreducing buffer (40% glycerol, 240 mM TRIS-HCl, pH 6.8, 8% SDS, 0.1% bromophenol blue) and loaded onto substrate polyacrylamide gels (25 μ l/lane). Substrate gels containing either bovine type B gelatin or bovine β -casein at a concentration of 2.8 mg/ml were used for zymography. Samples were run under nonreducing conditions in 7.5-10% substrate PAGE gels according to the procedure of Laemmli (1970). The gels were then washed 3 times for 10 min at room temperature in zymography wash buffer [50 mM TRIS-HCl (pH 7.5), 5 mM CaCl₂, 1 μ M

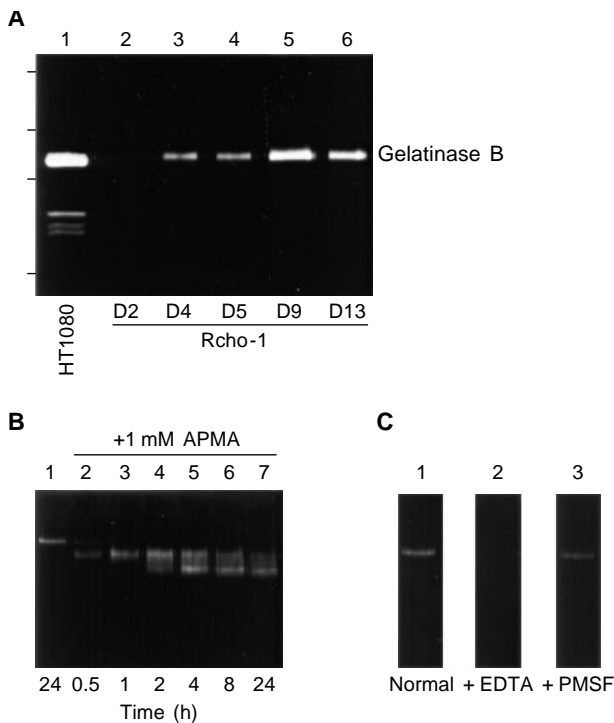


Fig. 3A–C Gelatin substrate gel analysis of Rcho-1 trophoblast-cell-conditioned media. **A** Stage-specific expression of gelatin-degrading enzymes. *Lane 1* conditioned medium from phorbol ester-stimulated treated human fibrosarcoma HT-1080 cells; *lanes 2–6* conditioned medium collected for 18 h after various stages of trophoblast differentiation (2–13 days of cell culture). **B** APMA activation of Rcho-1 trophoblast cell secreted MMP-9. Serum-free conditioned medium collected from differentiated Rcho-1 trophoblast cells (day 9 of culture) was activated with or without 1 mM APMA for 0.5–24 h at 37°C and run on a gelatin zymogram. *Lane 1* untreated day 9 Rcho-1 trophoblast-cell-conditioned culture medium incubated 24 h at 37°C; *lanes 2–7* day-9 Rcho-1 trophoblast-cell-conditioned culture media incubated at indicated times in the presence of 1 mM APMA. **C** Effects of EDTA and PMSF on Rcho-1 trophoblast cell secreted MMP-9 activity. Gelatin zymograms of HT1080 and day-9 Rcho-1 trophoblast-conditioned culture media incubated in the presence of a metalloproteinase inhibitor (5 mM EDTA) or a serine protease inhibitor (1 mM PMSF), respectively (*lane 1* no inhibitors added; *lane 2* incubated in EDTA; *lane 3* incubated in PMSF)

ZnCl₂, and 0.2% sodium azide] and incubated in zymography incubation buffer (zymography wash buffer + 2.5% Triton X-100) for 16–24 h at 37°C. Gels were then stained at room temperature for 30 min in Coomassie brilliant blue R-250 solution (20% methanol, 5% acetic acid, and 0.2% Coomassie brilliant blue R-250) and destained extensively in destain solution (20% methanol and 5% acetic acid). Lysed regions in the gel represent metalloproteinase activity. Prestained molecular weight standards and HT1080-conditioned culture medium containing human MMP-9 were used to estimate relative molecular weights of the clear lytic regions on substrate gels. At indicated times, proteinase inhibitors were added to the incubation buffer for the full incubation period at specified concentrations.

Northern blot analysis

Rcho-1 trophoblast cells were harvested at various stages of differentiation and processed for isolation of total RNA as described above. Total RNA separated on formaldehyde agarose gels (15 µg/lane) and lotted onto nylon membrane as previously described (Hamlin et al. 1994). Prehybridization was carried out at 42°C for 16 h in

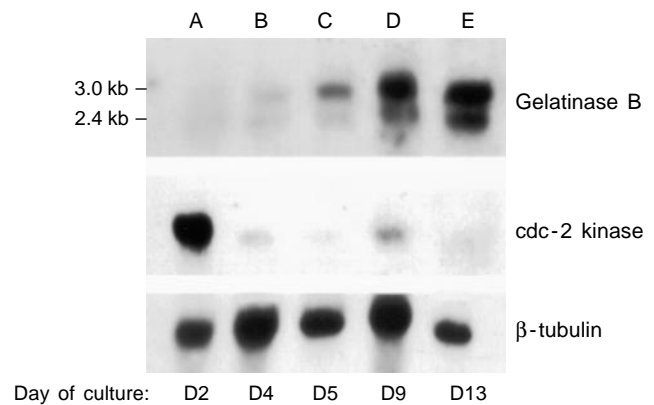


Fig. 4 Northern analysis of gelatinase B/MMP-9 and *cdc2* kinase mRNA in differentiating Rcho-1 trophoblast cells. Northern blot analysis of total RNA extracted from Rcho-1 trophoblast cells during proliferative (day 2) and various stages of differentiation (days 4–13). Fifteen micrograms of total RNA was run per lane and blots were probed with random prime labeled cDNAs for gelatinase B/MMP-9 (*top panel*), *cdc2* kinase (*middle panel*), or β -tubulin (*lower panel*)

prehybridization buffer (5×SSPE, 50% formamide, 5×Denhardt's, 0.5% SDS, and 100 µg/ml denatured salmon sperm DNA). Linearized cDNA probes were radiolabeled by random-priming and heat denatured. Hybridization was carried out for 16 h at 42°C in prehybridization buffer. Blots were washed twice for 15 min at 42°C in 4 × sodium saline citrate (SSC), 0.1% SDS and 1 time at 42°C in 1×SSC, 0.1% SDS for 30 min. Blots were exposed to X-ray film at –80°C. Ribosomal RNA was assessed for equal loading and general integrity of isolated total RNA. Hybridizations were performed with a radiolabeled cDNA to MMP-9 (Himmelstein et al. 1997) or alternatively with cDNAs to *cdc2* kinase and β -tubulin (Valenzuela et al. 1981), which were used as controls.

Western blot analysis

MMP-9 and *cdc2* kinase proteins were detected by Western blotting. MMP-9 was analyzed in medium conditioned by Rcho-1 trophoblast cells. During various stages of Rcho-1 trophoblast cell differentiation, conditioned culture medium was prepared by washing cells twice in HBSS and then incubating cells for 18 h in serum free NCTC-135 culture medium. Conditioned medium harvested from 12-*O*-tetradecanoylphorbol acetate-treated HT1080 cells cultured under serum-free conditions was used as a positive control for MMP-2 and MMP-9. Conditioned media were harvested, clarified by centrifugation, and concentrated via acetone precipitation. Pellets were collected by centrifugation, air dried, resuspended in water, and then mixed in nonreducing SDS-PAGE buffer. Samples were then separated in 7.5% polyacrylamide gels according to the procedure by Laemmli (1970) and electrophoretically transferred to nitrocellulose as previously described (Towbin et al. 1979). Nitrocellulose filters were then incubated overnight with shaking at 4°C in a blocking solution consisting of 5% nonfat dry milk in TRIS-buffered saline (TBS, 20 mM TRIS, pH 7.2, and 150 mM NaCl), followed by incubation with primary antibody, GeBC1-ID4 (Lyons et al. 1991), subsequent washes in fresh blocking solution for 16 h at 4°C, and three subsequent washes for 10 min each at room temperature in TBST (TBS with 0.5% Triton X-100). Filters were then incubated at room temperature in blocking solution for 1 h followed by a 2-h incubation at room temperature with blocking solution containing secondary antibody-enzyme conjugate. Western blots were then washed twice with shaking in TBST for 10 min each at room temperature. Immune complexes were detected by enhanced chemiluminescence (ECL) and exposed to X-ray film. *Cdc2* kinase was analyzed from Rcho-1 trophoblast cell lysates [lysis buffer: 10 mM TRIS-HCl, pH 7.5; 0.4 M NaCl; 1% Triton X-100,

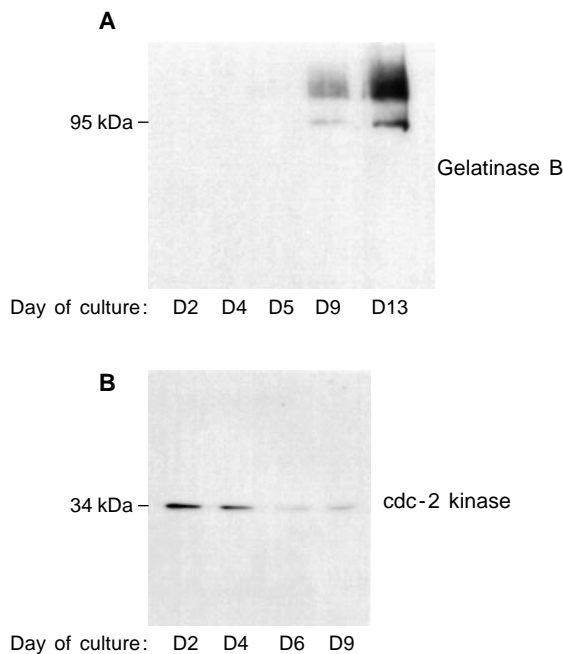


Fig. 5A, B Western blot analysis of gelatinase B/MMP-9 and *cdc2* kinase in differentiating Rcho-1 trophoblast cells. Serum-free conditioned medium was collected from Rcho-1 trophoblast cells at various stages of differentiation, immunoblotted, and probed with antibodies to rat gelatinase B/MMP-9 (**A**). Cell lysates were also isolated from differentiating Rcho-1 trophoblast cells, immunoblotted, and probed with antibodies to *cdc2* kinase (**B**)

0.1% NP-40 and 1 mM phenylmethylsulfonyl fluoride (PMSF)], separated in 12% polyacrylamide gels under reducing conditions, transferred to nitrocellulose, and probed with antibodies to MMP-9 immunoblotting.

MMP-9 promoter analysis

MMP-9 promoter/luciferase constructs were generated from a previously characterized 569-bp mouse MMP-9 promoter (Sato et al. 1993) using *Pfu* polymerase and PCR. Briefly, the 569-bp mouse MMP-9 promoter served as a template for PCR using an upstream primer with an *NdeI* restriction site DNA overhang 5' CTAGCTAGCTAGAAGGAGTCAGCCTGCTGGA 3' (sense, -569 to -550) and a downstream primer with a *BglIII* restriction site DNA overhang 5' CCGGAAGATCTCTGAGGACCGCAGCTT CTG 3' (antisense, -1 to +17). PCR was performed as previously described (Rasmussen et al. 1997): briefly, 30 cycles of PCR with the denaturing temperature at 94°C (1 min), annealing temperature at 50°C (1 min), and extension temperature at 72°C (1 min). The end product was digested with *NdeI* and *BglIII* and ligated into pGL2 luciferase reporter constructs. Sequence of the PCR-generated MMP-9 promoter was confirmed by DNA sequencing. DNA sequencing was performed using an Applied Biosystems Model 310 sequencer and Applied Biosystems Dye Terminator Cycle Sequencing kits (Foster City, CA).

Cells were transiently transfected with promoter-reporter DNA preparations using a liposome-mediated procedure (Orwig et al. 1997). Initially, cells were plated at 2×10^4 /well of a six-well plate. After 60 h, cells in each well were incubated with lipofectamine (10 μ l), 569-bp MMP-9 promoter/luciferase construct (2 μ g), and Opti-MEM (200 μ l) at 37°C for 7 h. The DNA/lipofectamine mixture was then removed and the medium was changed to NCTC-135 containing 10% HS. Cells were incubated for an additional 48 h and luciferase activity

was determined as per the manufacturer's instructions. Luciferase activity was normalized to protein concentration.

Stably transfected clones were derived after co-transfecting Rcho-1 trophoblast cells with 569-bp MMP-9/luciferase construct (5 μ g) and pSV2Neo (0.5 μ g). Selection was carried out for 2 weeks in the presence of geneticin (250 μ g/ml). Five clonal cell lines were established by limiting dilution, and luciferase activity was monitored for differentiation-dependent MMP-9 promoter activation.

Statistical analysis

The data were analyzed by analysis of variance. The source of variation from significant *F* ratios was determined with the Student Newman-Keuls multiple comparison test (Keppel 1973).

Results

Differentiation-dependent trophoblast cell invasion

Rcho-1 trophoblast cells were plated on matrigel-coated porous invasion chambers to determine whether they would invade extracellular matrix concurrent with their differentiation into trophoblast giant cells. Rcho-1 trophoblast cells first began invading through the matrigel support between 24 and 48 h of culture (Fig. 1). The number of cells that invaded increased as a function of time in culture (Fig. 1) and was positively correlated with trophoblast giant cell differentiation.

Integrin subunit expression in differentiating Rcho-1 trophoblast cells

Changes in trophoblast cell integrin expression have been correlated with acquisition of the invasive phenotype (Damsky et al. 1994). Previous work with mouse blastocyst outgrowths (Sutherland et al. 1993) had shown that $\alpha 1$ -integrin expression appeared only after trophoblast cells began to grow out, whereas $\alpha 5$ - and $\beta 1$ -integrin subunits were expressed continuously from egg stage to postimplantation. Accordingly, we set out to determine the stage-specific expression of integrin subunits $\alpha 1$, $\alpha 5$ and $\beta 1$ in Rcho-1 trophoblast cells by RT-PCR analysis. As predicted, the expression of $\alpha 5$ - and $\beta 1$ -subunits was expressed in both the proliferative and differentiated trophoblast cells, whereas the $\alpha 1$ -subunit was seen at higher levels in the differentiated trophoblast giant cells (Fig. 2).

MMP-9: the major metalloproteinase of differentiating Rcho-1 trophoblast cells

Gelatin zymograms showed that as trophoblast cells differentiated they predominantly secreted increasing levels of a 95-kDa gelatinase (Fig. 3A). The 95-kDa gelatinolytic activity migrated slightly higher than the latent or inactive MMP-9 secreted by human fibrosarcoma HT 1080 cells. When gelatinase zymograms were allowed to overdigest, a weak 72-kDa gelatinase was identified (presumably gelati-

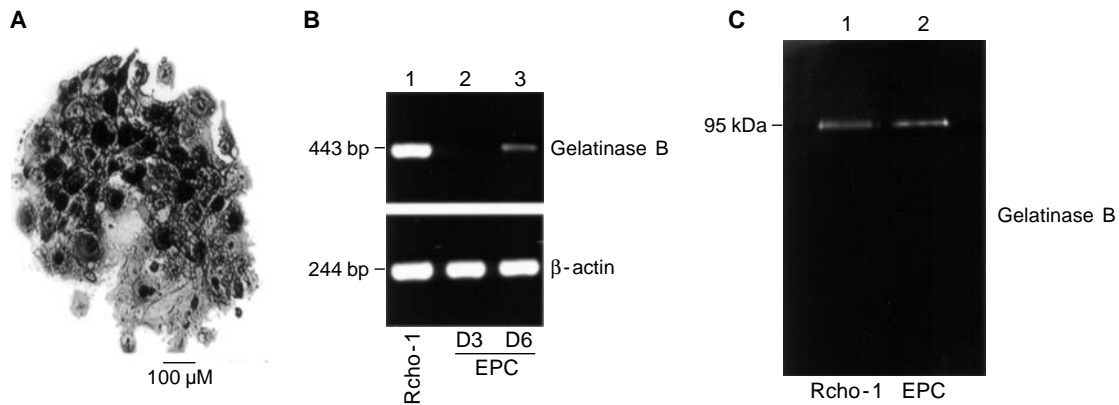


Fig. 6A–C Analysis of gelatinase B/MMP-9 in rat ectoplacental cone outgrowths. **A** A representative photomicrograph of a rat ectoplacental cone after 5 days of outgrowth. **B** Ectoplacental cone outgrowths and differentiated Rcho-1 trophoblast cells were analyzed by RT-PCR for expression of gelatinase B/MMP-9 (*upper panel*) or β -actin (*lower panel*). *Lane 1* Differentiated Rcho-1 trophoblast cells; *lane 2* day-3

ectoplacental cone outgrowths; *lane 3* day-6 ectoplacental cone outgrowths. **C** Gelatin zymography of metalloenzymes expressed by ectoplacental cone outgrowths and differentiated Rcho-1 trophoblast cells. *Lane 1* Differentiated Rcho-1 trophoblast-cell-conditioned medium; *lane 2* day-6 ectoplacental-cone-outgrowth-conditioned medium

nase A/MMP-2). Levels of this 72-kDa gelatinase did not change during trophoblast differentiation (data not shown). Casein zymography did not reveal any enzyme activity even when processed following tenfold concentration of Rcho-1 trophoblast-conditioned medium.

We next evaluated the nature of the trophoblast-cell-secreted 95-kDa gelatinase through examination of its sensitivity to metalloenzyme activators (aminophenylmercuric acetate, APMA) and inhibitors (ethylenediaminetetraacetic acid, EDTA). Activation of Rcho-1 trophoblast-cell-conditioned medium with APMA (1 mM) resulted in the conversion of 95-kDa gelatinase to its lower molecular weight activated form (Fig. 3B). Gelatin zymograms run in the presence of EDTA (5 mM) totally abolished gelatinolytic activity, whereas PMSF (1 mM, serine proteinase inhibitor) did not abrogate enzyme activity (Fig. 3C). Taken together, the 95-kDa gelatinase is the major MMP expressed in differentiating trophoblast cells and its overall properties are consistent with it being gelatinase B/MMP-9.

MMP-9 mRNA and protein expression in differentiating Rcho-1 trophoblast cells

Northern blot analyses were performed to evaluate MMP-9 expression in differentiating trophoblast cells. Two major transcripts were identified, a prominent 3.0 kb and a minor 2.4 kb that corresponded with previously reported rat MMP-9 mRNA species (Okada et al. 1995; Fig. 4, top panel). Both transcripts were identified at low levels in proliferating Rcho-1 trophoblast cells (day 2 of culture) and increased as the trophoblast cells differentiated (days 4–13 of culture). In contrast, *cdc2* kinase transcripts were high-proliferating trophoblast cells (day 2 of culture) and significantly diminished accompanying trophoblast cell differentiation (Fig. 4, middle panel). Levels of β -tubulin mRNA were relatively constant in both proliferative and differentiating trophoblast cells (Fig. 4, bottom).

Western blot analysis of Rcho-1 trophoblast-cell-conditioned medium identified a 95-kDa band at day 9 that further increased levels by day 13 of culture (Fig. 5A). Similar to the Northern blot analysis, levels of *cdc2* kinase protein were shown to decrease as cells differentiated into trophoblast giant cells (Fig. 5B).

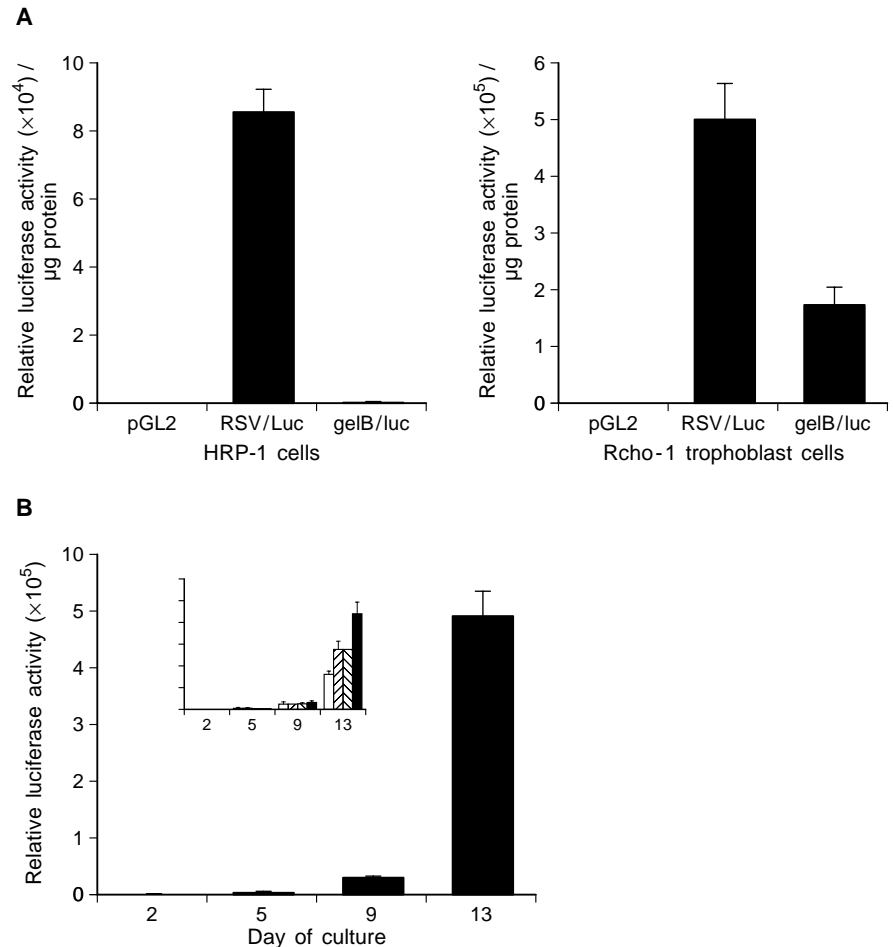
Primary cultures of rat ectoplacental cone outgrowths express MMP-9

Rat ectoplacental cone explants were cultured for 3 or 6 days and expression of MMP-9 was monitored. The morphology of trophoblast giant cells from day-6 ectoplacental cones is shown in Fig. 6A. MMP-9 detected independently from differentiating ectoplacental cones by RT-PCR (Fig. 6B) and/or gelatin zymography (Fig. 6C) was similar in relative size to species present in differentiating Rcho-1 trophoblast cells. Thus, differentiating ectoplacental cone cells express MMP-9 as they morphologically differentiate into trophoblast giant cells, similar to the behavior of differentiating Rcho-1 trophoblast cells.

Differentiation-dependent MMP-9 promoter activation

In order to determine whether the MMP-9 gene was regulated at a transcriptional level, MMP-9 promoter/reporter constructs were evaluated following both transient and stable transfection. A 569-bp MMP-9 promoter/luciferase reporter construct was found to be active in transiently transfected Rcho-1 trophoblast cells. The promoter/reporter construct exhibited approximately 35% of the activity of an RSV promoter/luciferase reporter construct in the Rcho-1 trophoblast cells (Fig. 7A). In contrast, the same MMP-9 promoter/reporter construct exhibited less than 1% of the activity of the RSV promoter/reporter construct in the HRP-1 trophoblast stem cell line (Fig. 7A). Please note that MMP-9 mRNA is not detectable in HRP-1 cells (B.M.

Fig. 7A, B Gelatinase B/MMP-9 promoter analysis in Rcho-1 trophoblast cells. **A** Transient transfection of trophoblast cells with a 569-bp gelatinase B/MMP-9/ promoter-luciferase reporter construct. Rcho-1 trophoblast cells or HRP-1 trophoblast stem cells were plated in six-well culture dishes at 2×10^4 and maintained in NCTC-135 culture medium +20% FBS for 60 h. A total of 2 μ g DNA (per well) was transfected into the cells using a liposome-mediated procedure. Cells were then maintained in serum free NCTC-135 supplemented with 10% HS for 48 h. Following the incubation, cells were lysed and luciferase activity determined. Relative luciferase activity was normalized for cellular protein content. **B** Stable transfection of Rcho-1 trophoblast cells with the 569-bp MMP-9 promoter/luciferase reporter construct. The graph shows luciferase activity during various stages of differentiation for one representative clonal Rcho-1 trophoblast cell line (*bottom*) and four separate additional clonal Rcho-1 trophoblast cell lines (*inset*) each stably transfected with the MMP-9 promoter/luciferase reporter construct. All experiments were performed in triplicate and repeated at least 3 times. Data are presented as the means \pm standard error of the means. Statistical comparisons were made between control and treated (**A**) or between proliferative (day 2) and differentiating (days 5, 9, and 13) (**B**) trophoblast cells ($P < 0.05$)



Chapman and M.J. Soares, unpublished data). Since the MMP-9 promoter was active in Rcho-1 trophoblast cells capable of trophoblast giant cell differentiation but not in another trophoblast lineage (HRP-1 cells), we next examined the effect of differentiation on MMP-9 promoter activation.

Stable transfection of Rcho-1 trophoblast cells with MMP-9 promoter/reporter constructs was performed to determine if the differentiation-dependent expression of MMP-9 was regulated at the transcriptional level. Five separate clonal lines were isolated. All clones showed a differentiation-dependent increase in MMP-9 promoter activity (Fig. 7B). A 75- to 300-fold induction of MMP-9 promoter activity was observed during the differentiation of Rcho-1 trophoblast cells from day 2 to day 13 of culture.

Discussion

In this report, we have investigated the invasive phenotype of trophoblast cells derived from a rat trophoblast cell line and some aspects of the invasive phenotype of cells derived from the ectoplacental cone. Both in vitro model systems exhibit a differentiation-dependent acquisition of MMP-9 expression. MMP-9 expression is associated with transcriptional activation of the MMP-9 gene and development of an

invasive phenotype. The latter is further characterized by the pattern of integrin gene expression and cellular movement through a biological matrix.

Trophoblast cells are responsible for redirecting the efforts of maternal tissues into structures that facilitate the growth and development of the embryo/fetus. This task is accomplished, at least in part, via trophoblast cells and their secretion of hormones/cytokines and proteases. Some actions are systemic involving specific alterations in tissue/organ function. The remaining actions are intrauterine where dramatic changes occur in cellular and extracellular compositions. In the rat and mouse, acquisition of trophoblast endocrine and invasive phenotypes is differentiation dependent and is associated with the same differentiated cell type. These activities are attributed to trophoblast giant cells located at the interface of maternal and extraembryonic structures (Soares et al. 1996; present study). In contrast, the differentiation of human trophoblast cells towards endocrine and invasive phenotypes represents different cellular lineages and distinct pathways (Bass et al. 1994; Ringler and Strauss 1990). Indeed, human trophoblast invasiveness is characteristic of mononuclear trophoblast cells arranged in columns prevalent during the first trimester of pregnancy, whereas endocrine activities are attributed to syncytial trophoblast cells that increase in number as gestation proceeds (Bass et al. 1994; Ringler and Strauss 1990). In both ro-

dents and primates, trophoblast cell invasion and uterine remodeling is related to increased production of MMP-9 (Librach et al. 1991; Behrendtsen et al. 1992; Canete-Soler et al. 1995; Reponen et al. 1995; Harvey et al. 1995; Leco et al. 1996; Alexander et al. 1996; Das et al. 1997; present study).

Regulation of trophoblast MMP-9 expression provides a potential means for limiting trophoblast invasiveness and allowing proper establishment of placental exchange between maternal and fetal compartments. In situ hybridization experiments have shown that trophoblast giant cell expression of MMP-9 is limited to times when active invasion or remodeling is occurring (Leco et al. 1996; T.J. Peters, B. Liu, and M.J. Soares, unpublished results). MMP-9 expression has also been shown to be temporally regulated during other normal physiological processes, including menstruation (Salamonsen 1994; Hulboy et al. 1997) and parturition (Lei et al. 1995). Although in situ expression of MMP-9 in rodents correlates with uteroplacental remodeling, the Rcho-1 trophoblast cell line expressed increasing levels of MMP-9 with no apparent decrease through 13 days of culture. This apparent divergence between in vivo and in vitro controls has also been seen in a differentiation-dependent endocrine marker, placental lactogen-I (PL-I), whose expression is suppressed in vivo after mid-gestation but not in differentiating Rcho-1 trophoblast cells. Removal of trophoblast cells from maternal or embryonic cue(s) may be responsible for preventing the cells from turning off their expression of these differentiation-dependent genes. Alternatively, this dysregulation may be an aberration inherent in Rcho-1 trophoblast cells.

The activation of MMP-9 gene expression in trophoblast cells is regulated at the transcriptional level and is differentiation dependent (present study). A promoter comprising 569 bp DNA flanking the MMP-9 gene was activated as trophoblast giant cells were formed. Other genes associated with the acquisition of the trophoblast giant cell endocrine phenotype are similarly transcriptionally activated. Regulatory DNA flanking the placental lactogen-I (PL-I), cytochrome P450 side-chain cleavage enzyme, prolactin-like protein-Cv, and decidua/trophoblast prolactin-related protein genes similarly direct trophoblast-specific and differentiation-dependent activation in trophoblast giant cells (Shida et al. 1993; Yamamoto et al. 1994; Dai et al. 1996; Orwig et al. 1997). More specifically, AP-1 and GATA elements within the PL-I promoter participate in the control of trophoblast-giant-cell-specific expression of the PL-I gene (Shida et al. 1993; Ng et al. 1994). Consensus AP-1 and GATA elements are present within the 569-bp MMP-9 promoter (Sato and Seiki 1993), and transcription factors (*fos*, *jun*, GATA-2, GATA-3) responsible for transactivation from these sites are expressed in Rcho-1 trophoblast cells (Shida et al. 1993; Ng et al. 1994). Additionally, work with the MMP-9 promoter in various human cancer cell lines or in a rat embryo cell line has shown that AP-1, NF- κ B, Sp-1, ets, and retinoblastoma-binding sites participate in the transcriptional regulation of the MMP-9 gene (Sato et al. 1993; Sato and Seiki 1993; Gum et al. 1996; Himelstein et al. 1997). Whether these putative regulatory elements within

the MMP-9 promoter participate in the trophoblast giant cell differentiation-dependent activation of the MMP-9 gene remains to be determined.

Model systems used for studying invasive properties of trophoblast cells are numerous and have been previously reviewed (Pijnenborg et al. 1981; Aplin 1991; Damsky et al. 1993; Cross et al. 1994; Lala and Hamilton 1996; Bischof and Campana 1997). While human primary cytotrophoblast cultures can show initial invasive behavior, they have been difficult to work with because they spontaneously differentiate toward an endocrine phenotype and lose their invasive attributes. Additionally, they present interpretational problems because they generally represent a mixed population of trophoblast and nontrophoblast cells. Commonly used human choriocarcinoma cell lines such as JAR, JEG-3 and BeWo have been used for some invasive studies, but they are reported to be weakly invasive and do not exhibit a differentiation-dependent invasive phenotype (Graham et al. 1994). Consequently, there are inherent difficulties in discerning whether the invasive phenotype of choriocarcinoma cells is associated with their transformed or trophoblast phenotypes. Some laboratories have used rodent primary trophoblast, blastocyst outgrowths or ectoplacental cone outgrowths for studying trophoblast cell invasive behavior (Sutherland et al. 1988; Babiarz et al. 1992; Yelian et al. 1993; Harvey et al. 1995; Suenaga et al. 1996; Tanaka et al. 1998); however, these preparations are available in limited amounts, and, as with human primary trophoblast cell preparations, they potentially contain mixed cell types and spontaneously differentiate. The Rcho-1 trophoblast cell line described in this study provides a useful alternative. Rcho-1 trophoblast cells can be readily expanded or differentiated in vitro. Invasion is differentiation-dependent and is characterized by many of the features associated with the invasive phenotype including the expression of MMP-9 (present study).

Acknowledgements We would like to thank Donna Millard, Christopher B. Cohick and Drs. Kyle E. Orwig, Donald C. Johnson, and Heiner Müller for their advice. We would also like to acknowledge Drs. Hening Birkedal-Hansen and Jeffrey A. Engler and Dr. Ruth Muschel for their generous gift of antibodies and a cDNA to MMP-9, respectively.

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