EGF-induced trophoblast secretion of MMP-9 and TIMP-1 involves activation of both PI3K and MAPK signalling pathways

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Abstract

Epidermal growth factor (EGF) is present in the maternal-fetal environment and has an important role in placental development. Matrix metalloproteinase-9 (MMP-9) expression/activation is a pre-requisite in extravillous trophoblast invasion. Whereas EGF up-regulates MMP-9 activity in a variety of cell types, there is no direct evidence for the stimulation of MMP-9 and tissue inhibitor of metalloproteinase-1 (TIMP-1) secretion by EGF in extravillous trophoblasts. In addition, the signalling pathways involved in this regulation are not clear. In the present study, we have examined the possible involvement of the phosphatidylinositol 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) pathways in the regulation of the MMP-9/TIMP-1 system by EGF *in vitro*. We used a well-established invasive extravillous trophoblast cell line (HTR8/Svneo) and measured gene and protein expression by semi-quantitative RT-PCR and western analysis respectively. MMP activity was determined by zymography. We showed for the first time that EGF activated both PI3K/Akt and MAPK/extracellular-signal regulated kinase (ERK) signalling in HTR8/SVneo, and increased both MMP-9 and TIMP-1 mRNAs and protein concentrations. Interfering with either signalling pathway via PI3K inhibitor LY294002 or MEK inhibitor U0126 in EGF-stimulated HTR8/SVneo cells blocked the induction of MMP-9 and TIMP-1. LY294002 inhibited Akt phosphorylation, but had no effect on ERK phosphorylation; U0126 suppressed ERK phosphorylation without interfering with the phosphorylation of Akt. In addition, expression of constitutively active Akt (Myr-Akt1, Myr-Akt2, Myr-Akt3) was not sufficient to induce proMMP-9 and TIMP-1 secretion. Our results suggest that the activation of both PI3K and MAPK pathways in extravillous trophoblasts is necessary for the up-regulation of MMP-9 and TIMP-1 expression by EGF.

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Introduction

Matrix metalloproteinases (MMPs) are a family of neutral proteinases that catalyse the destruction of the extracellular matrix (ECM). Among the MMPs identified, MMP-9 (gelatinase B) appears to have an important role in a wide array of physiological and pathophysiological processes, including placental development, wound healing, angiogenesis, inflammation and tumour invasion/metastasis (Van den Steen *et al.* 2002). Degradation and reconstitution of ECM in uterine endometrium are required for trophoblast invasion and are regulated by MMPs (Roth & Fisher 1999). In particular, MMP-9 is highly expressed by extravillous trophoblasts during trophoblast invasion in the first trimester of gestation (Behrendtsen *et al.* 1992). In *in vitro* studies, MMP-9 expression is crucial for trophoblast invasion into collagen gels. MMP-9 expression/activation is a pre-requisite and rate-limiting step in trophoblast invasion (Bischof *et al.* 2002, Campbell *et al.* 2003) and aberrant MMP-9

expression in extravillous trophoblasts is linked to pre-eclampsia, a disease characterized by poor trophoblast invasion (Kolben *et al.* 1996). MMP-9 knockout mice have impaired reproduction (Dubois *et al.* 2000). Thus studies of the mechanism regulating the expression of MMP-9 are important to an understanding of matrix degradation and extravillous trophoblast invasion.

MMP-9 secretion in invasive cytotrophoblasts is stimulated by interleukin (IL)-1 β (Librach *et al.* 1994), tumour necrosis factor α (TNF α) (Meisser *et al.* 1999), insulin-like growth factor binding protein (IGFBP)-1 (Bischof *et al.* 1998), leptin (Castellucci *et al.* 2000) and human chorionic gonadotropin (hCG) (Licht *et al.* 2001). In contrast, IL-10 (Roth & Fisher 1999) and transforming growth factor β (Meisser *et al.* 1999) are known to inhibit trophoblastic MMP-9 secretion. Epidermal growth factor (EGF)-induced MMP-9 secretion, believed to facilitate tumour invasion and metastasis, has been demonstrated in various tumour

cells (Ellerbroek *et al.* 1998, Charoenrat *et al.* 2000, Cox *et al.* 2000, Liu & Klominek 2003, Nutt *et al.* 2003). Although it has been shown that EGF stimulates trophoblast invasion (Bass *et al.* 1994), the influence of the growth factor on MMP-9 secretion by extravillous trophoblasts has not yet been reported.

Tissue inhibitors of metalloproteinases (TIMPs) are important regulators of MMP activity (Denhardt *et al.* 1993). Although several members of the TIMP family have been identified, proteolysis by MMP-9 is mostly regulated by the action of endogenous TIMP-1 (Itoh & Nagase 1995). In addition, TIMP-1 is a multifunctional protein, known to be involved in the control of cell growth, differentiation and programmed cell death, independent of its influence on MMP-9 (Guedez *et al.* 1998, Li *et al.* 1999).

It is well established in various systems that EGF receptor (EGFR) ligation leads to activation of the phosphatidylinositol 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) pathways, resulting in the activation of several transcription factors. For instance, EGFR activates the PI3K and MAPK/extracellular-signal regulated kinase (ERK) pathways, which modulate the activation of NF κ B and AP-1 respectively, in human head and neck squamous cell carcinoma lines (Bancroft *et al.* 2002). Transcription factors Ets-1 and -2 are activated in response to EGF in human breast tumour cells (Watabe *et al.* 1998). It has also been demonstrated that regulatory elements of the transcription factors AP-1, Ets and NF κ B are present in the promoter region of the MMP-9 gene (Huhtala *et al.* 1991), whereas the TIMP-1 promoter contains AP-1 and Ets binding sites (Borden & Heller 1997). Therefore, we hypothesized that EGF induces MMP-9 and TIMP-1 secretion through activation of PI3K and MAPK signalling in extravillous trophoblasts.

In this paper, we investigated the effect of EGF on the secretion of MMP-9 and TIMP-1 and the signalling pathways involved in the regulation of MMP-9 and TIMP-1 by EGF in the invasive trophoblast cell line TR8/SVneo. We found that EGF up-regulated the secretion of both MMP-9 and TIMP-1. Using pharmacological inhibitors and molecular approaches, we have shown that both PI3K/Akt and MAPK/ERK signalling pathways are essential to the increase in trophoblast secretion of MMP-9 and TIMP-1 by EGF. We conclude that EGF-induced increases in MMP-9 and TIMP-1 secretion require the activation of both PI3K and MAPK signalling pathways.

Materials and Methods

Cell lines and culture conditions

The HTR-8/SVneo cells were a gift provided by Dr Charles H. Graham (Queen's University, Kingston, ON, Canada). This cell line was established from explant culture of first-trimester human placenta and immortalized by the simian virus 40 large T antigen (Graham *et al.* 1993). These cells exhibit a high proliferation index and

share various phenotypic similarities with the parental HTR-8 cells, including *in vitro* invasive abilities. Cells (6×10^5 /well in six-well plates) were plated for 6 h in 1 ml RPMI 1640 and 10% fetal bovine serum (FBS), first cultured overnight and subsequently incubated in fresh serum-free media containing EGF (Sigma) in various concentrations (0, 0.1, 1, 10, 50 ng/ml). In experiments with pharmacological inhibitors, inhibitors of PI3K (LY294002, 20 μ mol/l; Sigma) or of MAPK (U0126, 10 μ mol/l; Cell Signalling, Beverly, MA, USA) were dissolved in DMSO and added to each group 1 h before treatment with EGF (10 ng/ml, 24 h). Each experimental group received an equal amount of DMSO (1:1000; vehicle). Whole cell lysates were subjected to Western blot analysis to determine the content of the phosphorylated form of EGFR, Akt and ERK1/2. Conditioned media were collected after 24 h of treatment for determination of MMP-9 and MMP-2 activity (zymographic analysis) and of the content of TIMP-1 and TIMP-2 (Western blot analysis). Total RNA was isolated and TIMP-1 and the abundance of MMP-9 mRNAs was determined by semiquantitative RT-PCR.

Western blot analysis

At the end of the culture period, cells were washed with ice-cold PBS and lysed by sonication in lysis buffer (50 mmol/l HEPES (pH 7.4), 150 mmol/l NaCl, 1 mmol/l EGTA, 10 mmol/l sodium pyrophosphate, 1.5 mmol/l MgCl₂, 100 mmol/l sodium fluoride, 10% glycerol, 1% Triton X-100, 1 mmol/l sodium orthovanadate, 1 mmol/l phenylmethylsulphonyl fluoride, 10 μ g/ml aprotinin). Insoluble material was removed by centrifugation (14 000 g, 4°C, 20 min) and the protein content in the supernatant was determined with the BioRad DC protein kit assay (BioRad). Aliquots of protein (50 μ g) were resolved by SDS-PAGE and electrotransferred to nitrocellulose membranes. The membranes were blocked (1 h, room temperature) in blotting (Tris-buffered saline at pH 7.6 with 0.05% Tween 20 (TBS-T) and 5% dehydrated non-fat milk). After rinsing with TBS-T, membranes were immunoblotted with antibodies to phospho-EGFR (Upstate, Lake Placid, NY, USA), phospho-Akt (Cell Signalling Technology Inc., Beverly, MA, USA), phospho-ERK1/2 (Cell Signalling), phospho-p70S6K (Thr³⁸⁹) (Cell Signalling) or GAPDH (loading control; Abcam Ltd, Cambridge, UK). The conditioned medium, concentrated from 0.50 ml to approximately 30 μ l with Microcon YM-3 (Millipore Corporation, Bedford, MA, USA), was resolved by SDS-PAGE under reducing conditions. Aliquots of 10 μ g protein were resolved by SDS-PAGE and electrotransferred to nitrocellulose membranes. The membranes were immunoblotted with antibodies to TIMP-1 and TIMP-2 (Chemicon, Temecula, CA, USA). The bands were visualized using ECL reagents and quantitated with Scion Image Software (Beta 4.0.2, Scion Corporation, Frederick, MA, USA).

Zymographic analysis

The activities of MMP-9 and MMP-2 in the spent media were determined by zymography. Briefly, aliquots of the media containing 1 µg protein were incubated (10 min, room temperature) with 1/3 sample volume of loading buffer (200 mmol/l Tris-HCl, 8% SDS, 0.04% bromophenol and 40% glycerol) and resolved on a 10% SDS polyacrylamide gel containing gelatin (0.25 mg/ml; Sigma). The gel was then washed (2.5% Triton, 1 h with four changes of wash solution) to remove SDS and incubated (36 h, 37°C) in a renaturing buffer (50 mmol/l Tris-HCl pH 7.5, 10 mmol/l CaCl₂, 150 mmol/l NaCl and 0.02% sodium azide). Gels were stained with Coomassie brilliant blue and destained in methanol/acetic acid (30%/10% v/v). Proteolytic activity was identified as a clear band on a blue background. The images were scanned and quantitative enzyme analysis was carried out using Scion Image Software.

RNA isolation and semiquantitative RT-PCR analysis

Total RNA was isolated with RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Aliquots of 1 µg total RNA were used for first strand cDNA synthesis in 20 µl reaction volume with 200 units M-MLV reverse transcriptase (Invitrogen). The purity of the RNA preparations was confirmed by the absence of PCR product in the samples that had not undergone RT reaction. Primer pairs for cDNA amplification (in the 5'-3' direction) were as follows: GGATGGGAAGTACTGGCGATTC (forward) and CACTTGGTCCACCTGGTCAAC (reverse) for human MMP-9; CGCTGACATCCGGTTCGTCTAC (forward) and GTGGACACTGTGCAGGCTTCAG (reverse) for human TIMP1; GGAATTCGAGCAAGAGATGG (forward) and CACCTTCACCGTTCCAGTTT (reverse) for human β-actin. The expected fragment lengths of MMP9, TIMP-1 and β-actin were 478, 431 and 629 bp respectively. After the linear range of PCR for each target gene had been determined, PCR amplification was performed on the Mastercycler (Brinkmann Instruments Inc., Westbury, New York, USA) using HotStarTaq DNA polymerase (Qiagen) for 25 cycles for TIMP-1 and β-actin and 40 cycles for MMP-9. HotStarTaq DNA polymerase was activated at 95°C for 15 min before the beginning of the cycle (94°C for 30 s for denaturing, 54°C for annealing and 72°C for extension). PCR products on 2% agarose gels were stained with ethidium bromide and visualized under u.v. transillumination. The ratios of MMP-9 and TIMP-1 to β-actin were determined with the use of a computerized densitometric imager.

Transient transfection

The cells were plated overnight in RPMI 1640 and 10% FBS. At 60–80% confluence, cells were transfected with constitutively active Akt1 (HA-Myr-Akt1-PCMV6), constitutively active Akt2 (HA-Myr-Akt2-pcDNA3.1), constitutively

active Akt3 (HA-Myr-Akt3ΔPH-pcDNA3.1) expression vectors (generously provided by Dr Jin Cheng, University of South Florida, Tampa, FL, USA) or control vectors (PCMV6 and pcDNA 3.1) separately, using Effectene Transfection Reagent (Qiagen) and according to the manufacturer's instructions. After incubation for 6 h, cells were washed and incubated in serum-free media for 18 h.

Statistical analyses

All experiments were performed at least three times, and values are given as the mean ± s.e. Data were analysed by ANOVA and Newman-Keul's multiple comparison tests. The level of significance was set at $P < 0.05$.

Results

Effect of EGF and inhibitors of PI3K and MAPK on phospho-EGFR, phospho-Akt and phospho-ERK1/2 contents

EGF treatment (10 ng/ml) increased phospho-EGFR, phospho-Akt and phospho-ERK1/2 contents in HTR8/SVneo cells within 15 min of treatment (Fig. 1). Addition of the PI3K inhibitor LY 294002 (20 µmol/l) 1 h before EGF treatment had no effect on phospho-ERK content, but completely suppressed the EGF-induced increase in phospho-Akt content. In contrast, the MAPK inhibitor U0126 (10 µmol/l) was ineffective with respect to phospho-Akt content, but markedly suppressed the increase in phospho-ERK content induced by the growth factor. The presence of both inhibitors attenuated increases in both phospho-Akt and phospho-ERK1/2 contents (Fig. 1). The increased phospho-EGFR content in response to EGF treatment was not affected by the presence of the inhibitors (Fig. 1). Taken together, these findings demonstrate that, under the current

Figure 1 Effect of EGF on phospho-EGFR (P-EGFR), phospho-Akt (P-Akt) and phospho-ERK1/2 (P-ERK1/2) contents in the absence or presence of LY294002 or U0126. HTR8/SVneo cells were starved in serum-free medium overnight and treated with EGF (10 ng/ml) for 15 min. LY294002 (20 µmol/l) and U0126 (10 µmol/l) were added 1 h before EGF treatment where indicated in the figure. Whole cell lysates were used for immunoblotting assay to detect P-EGFR, P-Akt and P-ERK1/2.

experimental conditions, the effect of the inhibitors were indeed specific to the signalling pathways concerned.

Possible involvement of PI3K and MAPK in the regulation of MMP-9 and TIMP-1 mRNA abundance

To define the possible regulatory role of EGF on MMP-9 and TIMP-1 mRNA levels, the trophoblast cells were cultured for 24 h with various concentrations of EGF (0, 0.1, 1, 10, 50 ng/ml). As seen in Fig. 2A, EGF increased the abundance of MMP-9 (478 bp) and TIMP-1 (431 bp) mRNAs in trophoblast cells in a concentration-dependent manner and observable increases were evident at 10 ng/ml or more. In order to examine the involvement of PI3K and MAPK signalling in up-regulating the abundance of MMP-9 and TIMP-1 mRNAs, cells were treated with EGF (10 ng/ml) for 24 h with or without 1 h pre-treatment with LY294002 (20 µmol/l) or U0126 (10 µmol/l), or both. The increases in mRNA abundance for TIMP-1 and MMP-9 elicited by the growth factor were inhibited in the presence of LY294002 or U0126, or both (Fig. 2B).

Effect of EGF and inhibitors of PI3K and MAPK on proMMP-9 activity and TIMP-1 content

Culture of trophoblast cells in the presence of EGF resulted in a concentration-dependent increase in the activity of proMMP-9 (92 kDa). In contrast, proMMP-2 activity (72 kDa) remained unchanged irrespective of the concentration of EGF (Fig. 3A).

To examine whether regulation of proMMP-9 activity involves PI3K or MAPK, or both, cells were treated with these specific inhibitors, as described above. In the presence of EGF (10 ng/ml), treatment with either LY294002 or U0126 markedly reduced the activities of proMMP-9, although the response was more pronounced with the PI3K inhibitor (Fig. 3B). The presence of both inhibitors elicited a greater response compared with that observed with EGF/U0126 treatment, but not with EGF/LY294002 treatment (Fig. 3B).

Western blot analysis of conditioned media after EGF treatment indicated a concentration-dependent increase in the content of TIMP-1, but not of TIMP-2 (Fig. 4A). Pre-treatment of trophoblast cells with LY294002 or U0126

Figure 2 Effect of EGF and inhibitors of PI3K and MAPK on the abundance of MMP-9 and TIMP-1 mRNA. (A) Concentration effect of EGF (0, 0.1, 1, 10 and 50 ng/ml) on MMP-9 and TIMP-1 mRNAs after treatment for 24 h. (B) Influence of inhibitors of PI3K (LY294002; LY, 20 µmol/l) and MAPK (U0126, 10 µmol/l) on EGF (10 ng/ml)-induced levels of MMP-9 and TIMP-1 mRNAs. Top panels in (A) and (B) show representative results of agarose gel electrophoresis of RT-PCR product, and bottom panels are graphic representations of the relative mRNA levels. The amplification for each gene was in the linear range. Values represent means $\pm$ S.E.M. of three independent experiments. * $P < 0.05$ or ** $P < 0.01$ compared with control; + $P < 0.05$, ++ $P < 0.01$ compared with EGF alone.

Figure 3 Effect of EGF and inhibitors of PI3K and MAPK on MMP activity. (A) Concentration effect of EGF (0, 0.1, 1, 10 and 50 ng/ml) on proMMP-9 and proMMP-2 activity after treatment for 24 h. Top panel: representative proMMP-9 and proMMP-2 zymogram. Bottom panel: graphic representation of relative MMP activity. (B) Influence of inhibitor of PI3K (LY294002; LY, 20 μ mol/l) and MAPK (U0126, 10 μ mol/l) on EGF (10 ng/ml)-induced secretion of MMP-9. Top panel: representative proMMP-9 zymogram. Bottom panel: graphic representation of relative proMMP-9 activity. The values are the means $\pm$ S.E.M. of three independent experiments. $P < 0.01$: **compared with control, ++ compared with EGF alone, ## compared with EGF+U0126.

decreased both basal and EGF-induced TIMP-1 protein content. In the presence of both inhibitors, TIMP-1 secretion was completely suppressed. The inhibitors failed to alter TIMP-2 protein content in the trophoblast cells (Fig. 4B).

Effect of Akt activation on proMMP-9 and TIMP-1 secretion

The influence of Akt activation on the secretion of MMP-9 and TIMP-1 was examined by investigating their content in spent media after the expression of three constitutively active Akt isoforms (Myr-Akt1, Myr-Akt2 and Myr-Akt3). All three Akt isoforms were highly expressed and phosphorylated in whole cell lysate as detected by an immunoblotting assay with HA-Akt and phospho-Akt antibody respectively (Fig. 5A). In order to determine whether expressed active Akt forms were functional or not, an Akt downstream target, phosphorylation of p70S6K(Thr³⁸⁹), was examined. Although the expression of all active Akt forms increased phospho-p70S6K (Thr³⁸⁹) content, none of them had any effects on proMMP-9 activities (Fig. 5B) and TIMP-1 protein content (Fig. 5C) in the conditioned media.

Discussion

The ability of a stimulus to induce MMP-9 expression is dependent on the integration of specific signal transduction pathways, which leads to cell type specific and stimulus specific activation of the MMP-9 gene. For instance, phorbol 12-myristate 13-acetate (PMA)-induced expression of MMP-9 in tumour cells has been reported to be mediated through p38MAPK activation (Simon *et al.* 2001). However, in endothelial cells the classical mitogenic Raf/MEK/ERK cascade, but not the p38MAPK pathway, is responsible for PMA-induced MMP-9 expression (Genersch *et al.* 2000, Park *et al.* 2003). Protein kinase (PKC) is involved in the up-regulation of MMP-9 gene transcription by TNF α in glioma cells (Esteve *et al.* 2002a), whereas TNF α -induced MMP-9 secretion is up-regulated through Raf/MEK/ERK cascade and PKC is not involved in endothelial cells (Genersch *et al.* 2000). The roles of PI3K-dependent signalling in the regulation of MMP-9 expression are not consistent in various cell types (Esparza *et al.* 1999, Thant *et al.* 2000, Esteve *et al.* 2002b). In the present studies, we have shown that EGF activates both PI3K and MAPK pathways, leading to the up-regulation of MMP-9 in this trophoblast cell line. Both PI3K inhibitor and MAPK inhibitor, alone or in combination, blocked EGF-induced expression of MMP-9 (Figs 2B, 3B), suggesting that MMP-9 transcriptional activation may require the cooperation of two or more transcription factors activated by distinct signalling transduction pathways. It remains to be determined whether these signalling pathways further activate NF κ B (Bancroft *et al.* 2002) and AP-1 (Gum *et al.* 1997) respectively and whether activation of these transcription factors

promotes MMP-9 gene transcription (Huhtala *et al.* 1991), both of which have been demonstrated in other cell types. Our results are consistent with the reports that both PI3K/Akt and MAPK/ERK are essential signals for activation of MMP-9 transcription by fibronectin in ovarian

cancer cells (Thant *et al.* 2000) and IL-1 β in a mouse fibroblast cell line (Ruhul Amin *et al.* 2003).

Although MMP-2 and MMP-9 have similar substrate specificities, differences exist in their expression in response to EGF. Consistent with other reports in various other cell lines (Charoenrat *et al.* 2000, Liu & Klominek 2003, Nutt *et al.* 2003), our results showed that EGF stimulates secretion of proMMP-9 but not of proMMP-2 (Fig. 3A). It has also been shown that IL-1 α , leptin, 12-o-tetradecanoylphorbol 13-acetate (TPA) and TNF α up-regulate MMP-9 expression but have no effect on MMP-2 activity in human cytotrophoblasts (Gonzalez *et al.* 2001,

Figure 5 Expression of a constitutively active Akt (Myr-Akt1, Myr-Akt2 and Myr-Akt3) is not sufficient to induce secretion of proMMP-9 and TIMP-1. HTR8/SVneo cells were transiently transfected with Myr-Akt1, Myr-Akt2 and Myr-Akt3 or their parent vectors for 6 h and incubated in fresh medium without serum for 18 h. (A) Protein contents of HA-Akt (HA), P-Akt, P-P70S6K (Thr³⁸⁹) and GAPDH were detected in whole cell lysate by immunoblotting. (B) Zymographic analysis of proMMP-9 and proMMP-2 activity in conditioned media. (C) Western blot analysis of protein content of TIMP-1 and TIMP-2 in conditioned media.

Figure 4 Effect of EGF and inhibitors of PI3K and MAPK on TIMP-1 and TIMP-2 protein content. (A) Concentration-dependent effects of EGF (0, 0.1, 1, 10 and 50 ng/ml) on TIMP-1 and TIMP-2 protein contents after treatment for 24 h. (B) Influence of inhibitors of PI3K (LY294002; LY, 20 μ mol/l) and MAPK (U0126, 10 μ mol/l) on EGF (10 ng/ml)-induced secretion of TIMP-1 and TIMP-2. Contents of TIMPs were determined with Western blot in condensed media. The values are the means $\pm$ S.E.M. of three independent experiments. $P < 0.01$: **compared with control, + compared with EGF alone, ##compared with EGF+U0126, ^compared with EGF+LY294002.

Bischof *et al.* 2003), possibly because of the presence of different promoter elements on the gelatinase genes (Huh-tala *et al.* 1991).

The functional activity of MMP-9 is dependent on the relative abundance of the protease and its natural inhibitor, TIMP-1. In the present study, we have shown that both TIMP-1 and MMP-9 are up-regulated in a similar fashion after EGFR activation. These findings could be explained by the fact that the promoter of MMP-9 and TIMP-1 contains AP-1 and Ets binding sites (Borden & Heller 1997) and these transcription factors can be activated by EGF (Watabe *et al.* 1998, Li *et al.* 2003). As both Ets and AP-1 families are required to control TIMP-1 gene expression, it has been suggested that TIMP-1 expression may be regulated through the co-ordination of several signal transduction pathways (Logan *et al.* 1996). Indeed, our current studies have shown that both PI3K and MAPK signalling pathways are involved in the regulation of TIMP-1 expression in HTR8/SVneo cells.

Although our current observation of a concomitant up-regulation of MMP-9 and TIMP-1 by EGF may appear physiologically contradictory, this may be explained by the multifunctional properties of TIMP-1. It is possible that the up-regulation of the inhibitor is necessary to prevent excessive trophoblast invasion and to ensure normal placental development. Alternatively, the capacity of TIMP-1 to inactivate MMP-9 may be attenuated by other extracellular proteases present in the microenvironment of the trophoblast. For instance, leucocyte elastase has been shown to inactivate TIMP-1 while allowing the conversion of proMMP-9 to its catalytically active form (Itoh & Nagase 1995). In addition, TIMPs may serve other cellular functions in addition to MMP-9 regulation, including control of cell growth and differentiation and programmed cell death (Guedez *et al.* 1998, Li *et al.* 1999), although the mechanism involved remains to be elucidated.

Akt activation is one of the major downstream events of PI3K signalling. Three mammalian isoforms (Akt1/PKB α , Akt2/PKB β and Akt3/PKB γ) have been identified (Chan *et al.* 1999) and, despite a high degree of structural identity and a similar activation process, they appear to have distinct physiological roles (Cho *et al.* 2001) and a tissue-specific pattern of expression (Brodbeck *et al.* 1999, Nakatani *et al.* 1999). To investigate if Akt activation alone can up-regulate the expression of MMP-9 and TIMP-1, we have examined in the present studies whether the expression of the three constitutively active Akt forms (Myr-Akt1, Myr-Akt2 and Myr-Akt3) would increase their expression. Although the contents of phospho-Akt and phospho-p70S6K (Thr³⁸⁹) (a downstream target of Akt) increased in all three experimental groups (Fig. 5A), they were ineffective in altering the secretion of proMMP-9 and TIMP-1, suggesting that activation of Akt by itself is not sufficient for the induction of these proteins and again leading support to the contention that more than one signalling pathway is necessary for proMMP-9 and TIMP-1 secretion in HTR8/SVneo cells.

In the present study, LY294002 or U0126 alone appeared to suppress the expression of MMP-9 and TIMP-1 relative to control. This could not be attributable to the overall cytotoxic effects of the kinase inhibitor, because MMP-2 activity (data not shown) and TIMP-2 protein contents (Fig. 4B), in addition to the abundance of β -actin mRNA, were unaffected. Alternatively, it is possible that the observed changes in 'basal' parameters reflect suppression by the inhibitors of the PI3K/Akt or MAPK signalling pathway activated by an endogenous autocrine factor (e.g. EGF or IGF-II) secreted by the trophoblast cells during the culture period.

In conclusion, we have demonstrated that EGF stimulation increases the abundance of MMP-9/TIMP-1 mRNA and proMMP-9 activity, in addition to TIMP-1 protein content, in HTR8/SVneo cells. Both PI3K/Akt and MAPK/ERK signalling are essential to regulate the expression of MMP-9 and TIMP-1 by EGF in HTR8/SVneo cells. Increased MMP-9 and TIMP-1 expression induced by EGF involve the co-ordinated regulation of both PI3K and MAPK signalling pathways.

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