Molecular Human Reproduction Vol.12, No.4 pp. 225–232, 2006
Advance Access publication March 6, 2006 doi:10.1093/molehr/gal023

Involvement of MAPK pathway in TNF-α-induced MMP-9 expression in human trophoblastic cells

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The aim of this article was to investigate the signalling pathways involved in metalloproteinase-9 (MMP-9) expression induced by tumour necrosis factor-α (TNF-α) in first-trimester trophoblastic cells. TNF-α-induced MMP-9 expression, secretion and activity were completely blocked by stress-activated protein kinase/jun kinase (SAPK/JNK) and Erk inhibitors (SP600 125 and U0126 respectively) but not by p38 mitogen-activated protein kinase (MAPK) inhibitors (SB203 580 and SB202 190). Stimulation of HIPEC 65 cells with TNF-α caused phosphorylation of JNK and extracellular signal-regulated kinase 1/2 (Erk1/2), with a peak after 20 min of treatment. Transcription factors nuclear factor-κB (NF-κB) and activator protein 1 (AP-1)-binding site were identified as the cis-elements involved in TNF-α activation as determined by electromobility shift assays. TNF-α-induced transactivation of NF-κB was inhibited by U0126, whereas TNF-α-induced transactivation of AP-1 was inhibited by SP600 125. Taken together, these results indicate that in trophoblastic cells, TNF-α probably activates two different pathways leading to MMP-9 expression: (a) Erk1/2 pathway which in turn initiates NF-κB activation and (b) SAPK/JNK pathway that activates AP-1.

Key words: AP-1/cytotrophoblastic cells/Erk1/2/JNK/MMP-9/NF-κB/TNF-α

Introduction

Cytotrophoblastic cells (CTB) of the human placenta proliferate, migrate and invade the pregnant uterus to allow implantation and placentation (Red-Horse *et al*., 2004). The invasive property of trophoblastic cells is due to their ability to secrete proteases. Serine proteases, cathepsin and metalloproteinases (MMPs) are involved in this invasive process (Fisher *et al*., 1985; Bischof *et al*., 1995; Divya Chhikara *et al*., 2002). Among the MMPs, MMP-9 and MMP-2 have been shown to mediate invasion of CTB or CTB cell line into matrigel (Fisher *et al*., 1985, 1989, Staun-Ram *et al*., 2004). Trophoblastic expression of MMP-2 and MMP-9 has been studied during the first trimester of pregnancy (Xu *et al*., 2000). It was shown that the level of expression of MMP-9 was very small as compared with MMP-2, suggesting that MMP-2, rather than MMP-9, plays an important role in human embryo implantation. After the eighth week, MMP-9 secretion increases gradually, while MMP-2 production declines during the same period Expression of MMP-9 coincided with the maximal invasive potential of CTB, suggesting that MMP-9 is implicated in the invasiveness of these cells (Fisher *et al*., 1985; Librach *et al*., 1991).

The invasive behaviour of CTB is limited in time and in space and could be mediated in an autocrine way by trophoblastic factors and in a paracrine way by uterine factors. It has already been suggested that tumour necrosis factor (TNF-α) could serve as an autocrine growth factor in choriocarcinoma cells and might thus facilitate proliferation of CTB (Yang *et al*., 1993). Since MMP-9 is instrumental to trophoblast invasion (Librach *et al*., 1991) and since TNF-α is a known regulator of MMP-9 (Meisser *et al*., 1999), it was interesting to investigate the signalling pathways involved in the regulation of MMP-9 by TNF-α in CTB.

TNF-α can activate three known mitogen-activated protein kinase (MAPK) cascades in mammalian cells: stress-activated protein kinase/jun kinase (SAPK/JNK), p38 MAPK and Erk/MAPK (Baud and Karin, 2001; Wajant *et al*., 2003). These cascades of kinases have different functions and can cross-react at several levels. The SAPK/JNK pathway is involved in the regulation of TNF-α-induced gene expression by phosphorylation of transcription factors mainly c-jun and ATF-2, leading to increased activity of activator protein 1 (AP-1). The p38 MAPK enhances also the function of AP-1 but mainly through other transcription factors such as Elk-1 or CREB, whereas the Erk/MAPK pathway leads essentially (but not exclusively) to enhancing the function of nuclear factor-κB (NF-κB).

MAPK signalling pathways leading to increased expression of MMP-9 have already been studied in different TNF-stimulated cells (Holvoet *et al*., 2003; Moon *et al*., 2004; Wiehler *et al*., 2004). It was shown that TNF-α induced the secretion of MMP-9 from eosonophiles via p38 MAPK but not the extracellular signal-regulated kinase 1/2 (Erk1/2) pathway (Wiehler *et al*., 2004). In contrast, TNF-α-induced MMP-9 expression in keratinocytes involved the Erk1/2 cascade and AP-1 activation but no p38MAPK nor SAPK/JNK activation (Holvoet *et al*., 2003).

Since the AP-1 and NF-κB are known regulators of MMP-9 promoter (Van den Steen *et al*., 2002), this article investigates the signalling pathways induced by TNF-α that regulate MMP-9 expression in human trophoblastic cells.

Materials and methods

Reagents

Dulbecco's modified Eagle's medium high glucose (DMEM), Ham's nutrient mixture F-12 (Ham F12) and antibiotic mixture (penicillin and streptomycin)

were products of Invitrogen (Basel, Switzerland). Fetal bovine serum was from Biochrom AG (Oxoid AG, Basel, Switzerland). TNF was from R&D Systems (Buehlmann AG Basel, Switzerland). Tocriset MAPK inhibitor was from Tocris (Avonmouth, UK). Lysis buffer 5× was from Promega (Catalys AG, Wallisellen, Switzerland). BIO-RAD protein assay, Trans-Blot transfer medium were from Bio-Rad (Munich, Germany). Hybond-N+ membrane, Rainbow-stained protein molecular weight markers and enhanced chemiluminescence (ECL) western blotting detection system were from Amersham Biosciences (Buckinghamshire, UK). Electromobility shift assay (EMSA) and biotin 3´-end DNA-labelling kits were from Pierce (Rockford, IL, USA). Rabbit polyclonal phospho-SAPK/JNK, SAPK-JNK, phospho-p38MAPK, p38MAPK, phospho-Erk1/2, Erk1/2 antibodies and p38 MAPK control cell extracts were from Cell Signaling (Beverly, MA, USA). Sheep polyclonal MMP-9 antibody was from The Binding Site (Birmingham, UK). Goat polyclonal GAPDH-specific antibody was from Santa Cruz (Santa Cruz, CA, USA). The NF-κB and AP-1-specific oligonucleotides (5´-CCAGTGGAATTCCCCAGCCT-3´ and 5´-GAAGCTGAGTCAAAGAAGGC-3´ respectively) were purchased from Mycrosynth GmbH (Balgach, Switzerland).

Cell culture

The human invasive, proliferative extravillous cytotrophoblast cell line (HIPEC) 65 (a generous gift from Pr. D Evain-Brion, Paris; Pavan *et al*. 2003) and first-trimester CTB (isolation as described elsewhere, Bischof *et al*., 1995) were grown in DMEM high glucose/Ham F-12 (1/1) containing 10% fetal bovine serum (FBS) and antibiotics (100 U/ml penicillin, 100 µg/ml streptomycin) at 37°C in a humidified, 5% CO_2 atmosphere. Consent was obtained for the use of discarded tissue for research purposes using procedures approved by the Geneva Hospital Ethics Committee.

Effect of MAPK inhibitors on MMP-9 expression and secretion

HIPEC 65 and CTB were seeded in 12-well plates. One day later, they were cultured for 24 h in a serum-free medium before being treated with/without inhibitors (100 nM SB203 580, 100 nM SP600 125, 100 nM SB202 190 or 500 nM UO126). TNF-α (100 ng/ml) was added 1 h after the inhibitors and the cells incubated for 48 h. Culture supernatants were collected for analysis and cells washed with ice cold PBS, lysed with lysis buffer ×1, scraped and centrifuged at 7600 *g* for 10 min at 4°C.

Phosphorylation kinetic of p38MAPK, JNK and Erk1/2

HIPEC 65 cells were seeded in 6-well plates. After 24 h, the cells were cultured for 24 h in a serum-free medium before being treated for 1 h with or without inhibitors (same as above). TNF-α (100 ng/ml) was then added for 0, 10, 20, 30 and 60 min. Culture supernatants were collected for analysis and cells washed with ice cold PBS. Then, they were lysed with lysis buffer ×1, scraped, centrifuged at 7600 *g* for 10 min at 4°C and supernatants stored at –20°C until western blot analysis.

Zymography

Proteolytic activity of culture supernatants were assayed using gelatin-substrate gel electrophoresis as described previously (Martelli *et al*., 1993). Zymograms were scanned with an Epson Perfection 1 200 Photo scanner, and the surface of the digestion bands was measured by the Kodak 1D Image analysis software (Kodak, Rochester, NY, USA).

ELISA for MMP-2 and MMP-9

MMP-2 and MMP-9 concentrations were measured in the cell supernatants using our own enzyme immunoassays (EIA) as described and validated elsewhere (Meisser *et al*., 1999).

Western blot analysis

Protein concentration of cell extracts was determined by the Bio-Rad protein assay according to the instructions of the manufacturer. Proteins (50 µg) were denatured and subjected to SDS-PAGE using a 10% running gel. Rainbow-stained molecular weight markers were used as standards. Proteins were electrotransferred to nitrocellulose membranes. Nonspecific binding was blocked for 30 min at 37°C with 5% powdered milk in 0.2% NP40 buffer. MMP-9-specific polyclonal antibody (1/1000), GAPDH-specific antibody (1/750), phospho-JNK, phospho-p38 or phospho-Erk1/2-specific antibodies (1/800) and p38 or Erk1/2-specific antibodies (1/1000) were incubated overnight with the nitrocellulose membrane. After washing, the membranes were incubated with the appropriate horse-radish peroxidase (HRP)-linked secondary antibody (2 h, room temperature). After washing, the bands were revealed by chemiluminescence (ECL detection kit). Films were scanned with an Epson Perfection 1 200 Photo scanner, and the surface of bands was measured by the Kodak 1D Image analysis software (Kodak).

Preparation of nuclear extracts and EMSA

HIPEC 65 cells were plated in 25 cm^2 flasks and made quiescent at confluence by incubation in serum-free medium for 24 h. Cells were then incubated with or without TNF-α (100 ng/ml) at 37°C for 48 h. When inhibitors (SP600 125 or U0126) were used, they were added 1 h prior to the TNF-α treatment. After incubation, cells were washed twice with ice-cold PBS, then lysed with 0.5 ml of Hepes buffer pH 7.9 (10 mM Hepes, 10 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.5 mM PMSF, 0.4% NP40 and Roche protease inhibitor cocktail) for 10 min at room temperature. Cells were scraped and transferred to a tube placed on ice before being centrifuged at 4°C at 15 000 *g* for 3 min. The resultant pellet was resuspended in extraction buffer (20 mM Hepes, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 10% glycerol, 1 mM DTT, 0.5 mM PMSF and Roche protease inhibitor cocktail) and vigorously shaken at 4°C for 2 h on a shaking platform. The nuclear extracts were centrifuged at 4°C, 15 000 *g* for 5 min, and their protein concentration was determined before being aliquoted and stored at –80°C.

Biotin 3´-end DNA labelling was performed according to the instructions of the manufacturer. Binding reactions were conducted by incubation of 6 or 4 µg of nuclear protein from HIPEC 65 with 20 fmol of biotin end-labelled AP-1 or NF-κB probes respectively at room temperature for 20 min according to the instructions of the manufacturer. Subsequently, reaction mixtures were separated on a native 6% polyacrylamide gel and electrotransferred to nylon membranes at 4°C. The membranes were then cross-linked on a transilluminator for 15 min before detection of biotin-labelled DNA by chemiluminescence.

Invasion assay

Cell invasion assay was performed in an invasion chamber based on the Boyden chamber principle. Each insert is fitted with an 8 µM pore size polycarbonate membrane (Costar, Corning, NY, USA) precoated with rat tail collagen I (5 $\mu g/cm^2$). Inserts were washed in DMEM and incubated for 30 min at room temperature. For each well, 15×10^4 cells in 100 µl of serum-free media, in the presence or absence of TNF-α (100 ng/ml), were added to the upper compartment of the transwell chambers. Five hundred microlitres of media was added to the lower chamber. Cells were incubated for 48 h at 37°C in a CO_2 (5%) incubator. After incubation, the supernatant was discarded, and viable cells were stained with Chemicon cell stain (collagen-based cell invasion assay, Chemicon, Temecula, CA, USA). After washing, the stain was extracted with a solution of 1% acetic acid : 50% ethanol for 15 min at room temperature. Hundred microlitres of the dye mixture was transferred to a 96-well microtitre plate for colorimetric measurement at 560 nm. Data are expressed as the percentage of TNF-α-treated cells that invaded the collagen-coated membrane relative to the untreated (controls) cells.

Analysis of data

Data are expressed as the mean ± SEM and analysed with a Student's *t*-test at a $P < 0.05$ level of significance.

Results

Effect of TNF on 8–9 weeks CTB invasiveness

Invasion assays performed on 8–9 weeks CTB in the presence or absence of TNF-α (Figure 1) showed that addition of this cytokine increases relative invasion of these cells (about 42%, $P = 3 \cdot 10^{-6}$). Since MMP-9 has been shown to mediate invasion of CTB in matrigel and since TNF-α is a known regulator of MMP-9, we next investigated the signalling pathways involved in the regulation of MMP-9 by TNF-α in CTB and HIPEC 65 cell line.

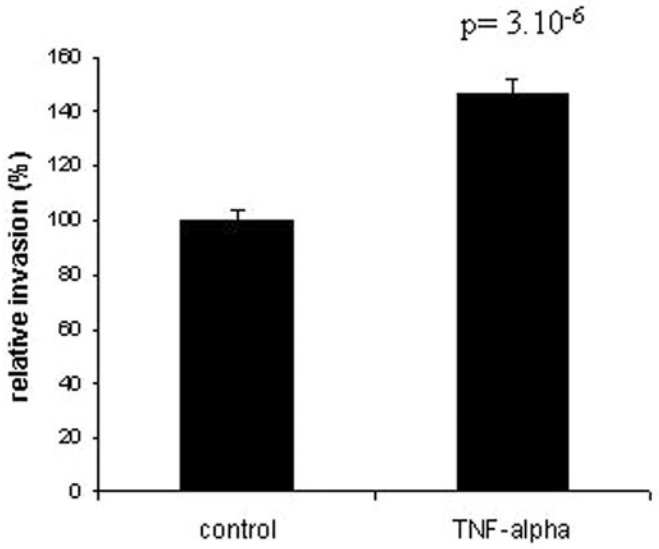

Figure 1. Increased *in vitro* invasion of 8–9 weeks cytotrophoblastic cells (CTB) by exogenous tumour necrosis factor-α (TNF-α). Primary cell invasion assay was performed in an invasion chamber based on the Boyden chamber principle. Cells were added in 100 µl of serum-free media, in the presence or absence of TNF-α (100 ng/ml), to the upper compartment of the transwell chambers. Media was added to the lower chamber. Cells were incubated for 48 h at 37°C in a CO_2 (5%) incubator. After incubation, the supernatant was discarded, and viable cells were stained with Chemicon cell stain. After washing, the stain was extracted with a solution of 1% acetic acid : 50% ethanol for 15 min at room temperature. Hundred microlitres of the dye mixture was transferred to a 96-well microtitre plate for colorimetric measurement at 560 nm. Experiment was performed three times with different cell preparations and samples run in triplicate. Statistics were performed with Student's *t*-test. Data are expressed as the mean ± SEM and analysed with a Student's *t*-test.

Inhibition of TNF-α-induced increase in MMP-9 activity and expression by MAPK inhibitors in trophoblastic cells

Treatment of trophoblastic cells (HIPEC65, Figure 2 and CTB, Figure 3) with TNF-α induced a two-fold increase of MMP-9 activity (Figures 2A and 3A), secretion (Figure 3B) and expression (Figure 2B), whereas the activity of MMP-2 was not modified (Figure 2A). To examine whether Erk1/2, JNK and p38 MAPK activations are involved in the signal transduction pathway leading to MMP-9 expression caused by TNF-α, different MAPK inhibitors were added 1 h before TNF-α treatment in HIPEC 65. While pretreatment of HIPEC 65 with p38 MAPK inhibitors (SB203 580 and SB202 190) had no marked effect on TNF-α-induced MMP-9 expression and activity (Figure 2), inhibitors of JNK and MEK, SP600 125 and U0126 respectively, decreased significantly the TNF-α-induced MMP-9 activity (P = 0.003 and $P = 4 \times 10^{-5}$ respectively) and expression (P = 0.03 and P = 0.02 respectively), suggesting that JNK and MEK but not p38 MAPK are implicated in these responses to TNF-α (Figure 2). Since similar results were obtained from primary CTB (Figure 3), HIPEC 65 represents a good model for TNF-α signalling studies in extravillous CTB.

Since the activation of Erk1/2 and JNK requires phosphorylation, western blots were performed to examine Erk1/2 and JNK phosphorylation using specific antibody for phospho-Erk1/2 and phospho-JNK. p38 MAPK phosphorylation induced by TNF-α was also investigated. HIPEC 65 cells were stimulated with TNF-α (100 ng/ml) for various periods of time (10, 20, 30 and 60 min). While p38 MAPK protein was present in HIPEC 65 and phospho-p38 MAPK was detected in positive control extracts, phospho-p38 MAPK was not detected in extracts from TNF-α-stimulated cells (Figure 4A). This result confirms that p38 MAPK is not activated by TNF-α and consequently is not involved in the signal transduction pathway leading to MMP-9 expression induced by TNF-α. When cells were stimulated with TNF-α, JNK phosphorylation began at 10 min, peaked at 20 min and then gradually decreased after 30 min of treatment (Figure 4B). Pretreatment with JNK inhibitor, SP600 125 (10 nm), 1 h before TNF-α treatment, delayed and decreased phosphorylation of JNK. The protein level of JNK protein was not affected by TNF-α addition. Treatment of HIPEC 65 cells with TNF-α also caused Erk1/2 activation (Figure 4C). This activation began at 10 min, with a maximum effect at 20 min and then gradually declined after 30 min of treatment. The protein level of Erk1/2 was not affected by TNF-α addition. When cells were pretreated with U0126 (50 nm) 1 h before TNF-α treatment, TNF-α-induced activation of Erk1/2 was markedly inhibited.

Involvement of AP-1 and NF-κB in TNF-α-induced MMP-9 expression

To elucidate whether AP-1 and NF-κB are involved in the TNF-α-induced signal transduction pathway leading to MMP-9 expression, binding of nuclear protein to AP-1 or NF-κB-specific DNA consensus sequences was estimated by EMSA. As shown in Figure 5, TNF-α induced the activation of AP-1 (compare lanes 3 and 4). This activation was completely inhibited in the presence of SP600 125 (lane 5), in contrast to in presence of U0126, and specific to AP-1 (lane 1). In the absence of nuclear proteins (lane 2), no chemiluminescence was observed, showing that only biotin-labelled DNA-binding proteins were detected. These results suggest that JNK is involved in TNF-α-induced activation of AP-1.

Treatment with U0126 (lane 6 compared to lane 4, Figure 6), but not SP600 125 (lane 5 compared to lane 4, Figure 6), inhibited the TNF-α-induced increase in NF-κB-binding activities, suggesting that NF-κB is involved in the Erk1/2 pathway leading to TNF-α-induced MMP-9 expression.

Discussion

TNF-α was first identified as a product of activated macrophages. It is now known that TNF-α is produced by many types of cells and could serve as an endogenous modulator of normal tissue homeostasis and physiology (Tovey, 1989; Hunt *et al*., 1992). Many studies showed that protein and TNF transcripts are present in human placental trophoblast cells (Chen *et al*., 1991; Haynes *et al*., 1993; King *et al*., 1995). Two types of receptor were described for TNF, and both are identified in choriocarcinoma cell lines and in lysates of human term placentas. These observations suggest that TNF could play a regulatory role in trophoblast invasiveness (Yang *et al*., 1993). This property could be due in part to its stimulating effect on MMP-9 activity in CTB (Meisser *et al*., 1999). However, in contrast to these considerations, it was observed that in first-trimester placental explant cultures (Bauer *et al*., 2004) or in the trophoblast cell line HTR-8/SVneo (Renaud *et al*., 2005), exogenous TNF decreased trophoblast invasiveness. In the present study, we observed that exogenous TNF-α increased invasion of 8–9 weeks CTB. This discrepancy suggests that trophoblastic cell lines do not respond in the same way as primary CTB. Trophoblastic cell lines are very useful models, but results obtained with these cells should always be verified with primary cells.

MMP-9 has been observed to be instrumental to trophoblast invasion (Librach *et al*., 1991); in this article, we show that treatment of CTB or a trophoblast cell line (HIPEC 65) by TNF-α induces a two-fold increase in MMP-9 expression, secretion and activity via the Erk1/2 and SAPK/JNK pathways. The results obtained from HIPEC 65 cells and CTB are very similar, and we may conclude that results

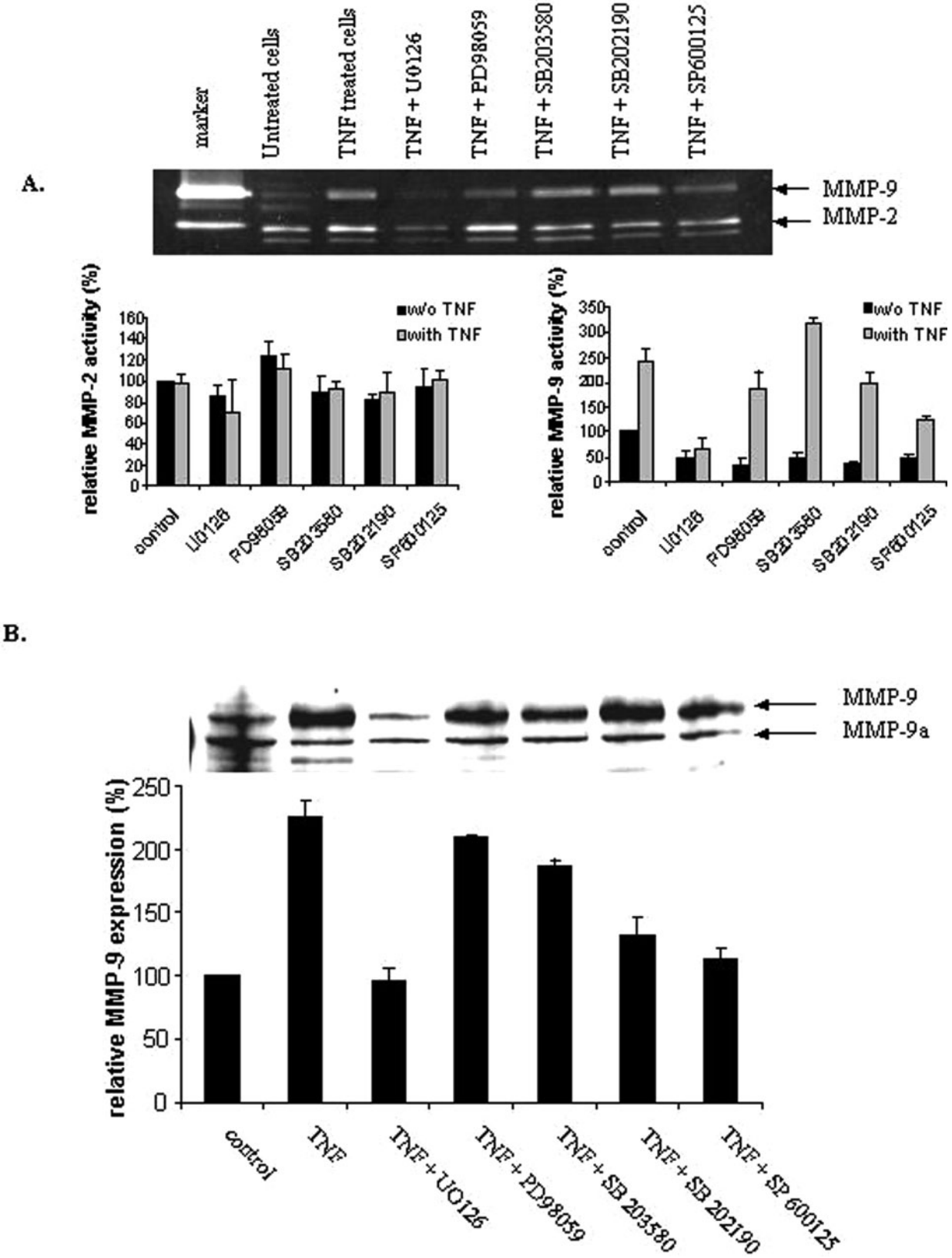

Figure 2. Down-regulation of tumour necrosis factor-α (TNF-α)-induced metalloproteinase-9 (MMP-9) activity and expression by mitogen-activated protein kinase (MAPK) inhibitors in HIPEC 65. Cells were treated with or without TNF-α (100 ng/ml) for 48 h. When inhibitors were used, they were added 1 h before TNF-α treatment. After incubation, culture supernatants were subjected to gelatin-substrate gel electrophoresis (**A**) and cell lysates to 10% SDS–PAGE and transferred to nitrocellulose membrane. Western blot analysis was performed using an MMP-9-specific polyclonal antibody (**B**). Bands of western blot visualized by an ECL method and surface of the digestion bands of zymogram were scanned and quantified by the Kodak 1D image analysis software. The results are expressed as the ratio of MMP-9 or MMP-2 band intensity of treated cells versus that of untreated cells used as control. Each experiment was performed two times with different cell preparations and samples run in triplicate. Statistics were performed with Student's *t*-test. $*P < 0.05$, as compared with TNF-α-treated control cells.

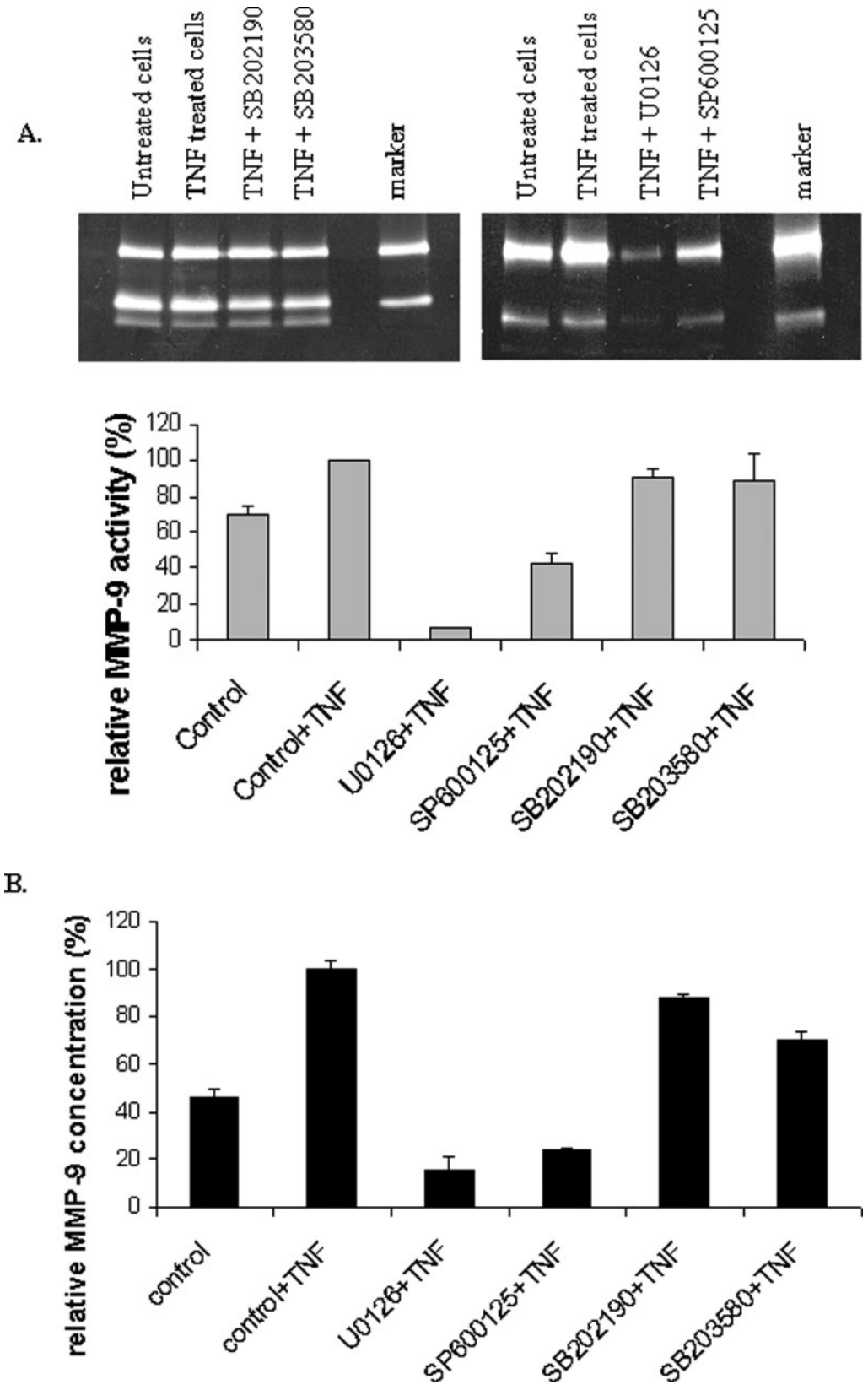

Figure 3. Tumour necrosis factor-α (TNF-α)-induced down-regulation of secreted metalloproteinase-9 (MMP-9) concentration and activity by mitogen-activated protein kinase (MAPK) inhibitors in cytotrophoblastic cells (CTB). Cells were treated with/without TNF-α (100 ng/ml) for 48 h. When inhibitors were used, they were added 1 h before TNF-α treatment. After incubation, culture supernatants were subjected to gelatin-substrate gel electrophoresis (**A**) or quantified by ELISA (**B**). Experiment was performed two times with different cell preparations and samples run in triplicate. Statistics were performed with Student's *t*-test. **P* < 0.05, as compared with TNF-α-treated control cells.

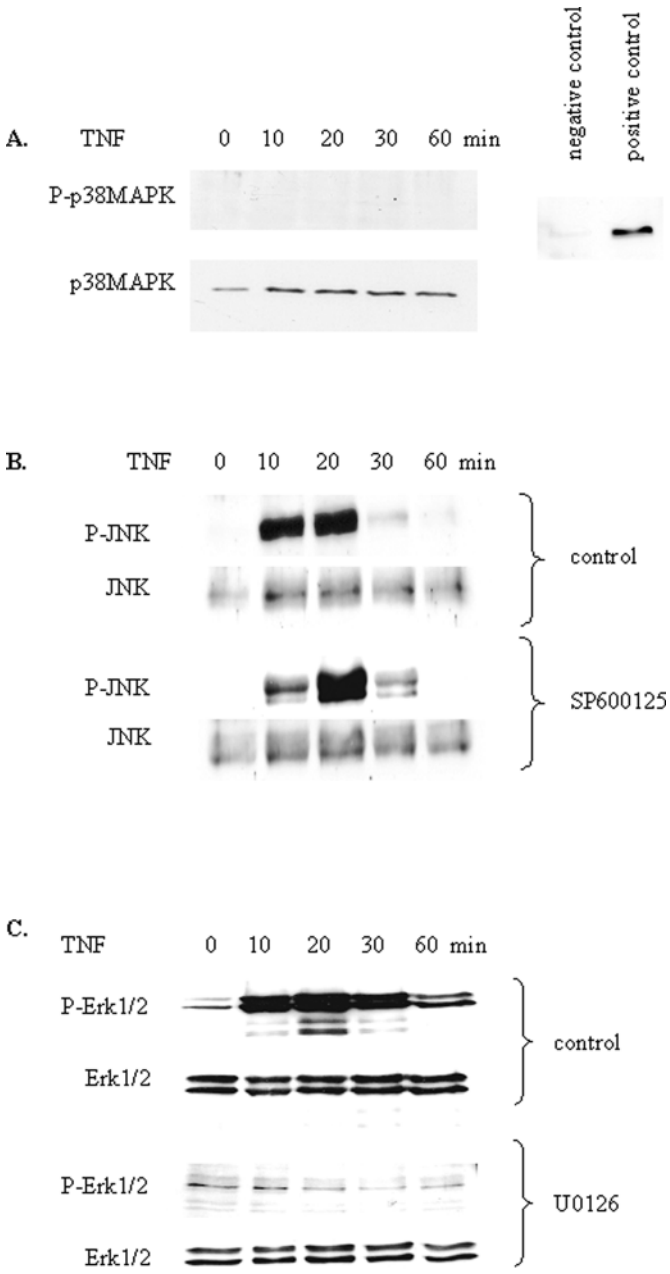

Figure 4. Erk1/2 and JNK, but not p38 mitogen-activated protein kinase (MAPK), are involved in tumour necrosis factor-α (TNF-α)-induced metalloproteinase-9 (MMP-9) expression in HIPEC 65 cells. (**A**) Cells were treated with TNF-α (100 ng/ml) for different time intervals, and p38 phosphorylation was evaluated by immunoblotting with an antibody specific for phosphorylated p38 (P-p38 MAPK). Equal loading in each lane is shown by the similar intensities of p38 MAPK. Negative and positive cell extract controls of phosphorylated p38 MAPK from Cell Signaling were used to confirm the specificity of anti-phosphorylated p38 MAPK antibody. (**B**) Cells were pretreated with SP600 125 or not for 1 h before TNF-α treatment for various periods of times. Effect of MAPK inhibitor on TNF-α-induced JNK phosphorylation was shown by immunoblotting with an antibody specific for phosphorylated JNK (P-JNK). (**C**) Cells were pretreated or not with U0126 for 1 h before TNF-α treatment for various periods of times. Effect of U0126 on TNF-α-induced Erk1/2 phosphorylation was shown by immunoblotting with an antibody specific for phosphorylated Erk1/2 (P-Erk1/2). Equal loading in each lane is shown by the similar intensities of Erk1/2.

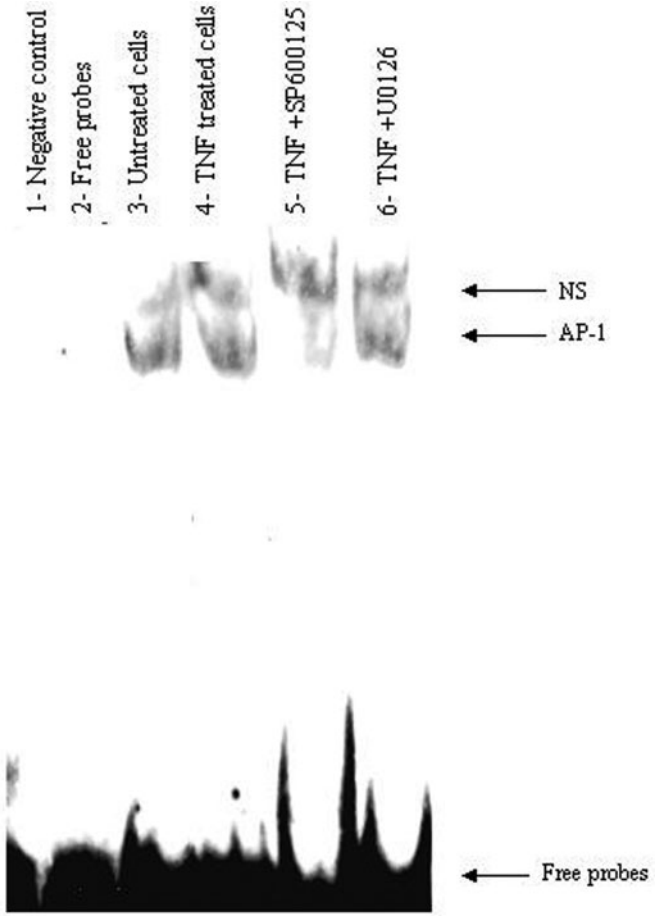

Figure 5. Nuclear factor-κB (NF-κB) DNA-binding capacity in TNF-α-stimulated HIPEC 65 cells. In the absence of nuclear protein (lane 2), no chemiluminescence was detected, demonstrating that only biotin-labelled DNA-binding proteins were detected. Unlabeled activator protein 1 (AP-1) consensus oligonucleotides (lane 1) were added in excess (4 pmoles) for specific competitive assay with biotin end-labelled AP-1 oligonucleotides. NS, nonspecific binding.

obtained from HIPEC 65 cells could correspond to the *in vivo* situation of extravillous CTB.

The signalling pathway of TNF-induced MMP-9 expression has already been studied in a number of cell types (Holvoet *et al*., 2003; Moon *et al*., 2004; Nee *et al*., 2004; Wiehler *et al*., 2004) but never, to the best of our knowledge, in trophoblastic cells. The study of Wiehler *et al*. (2004) showed that TNF-α stimulates MMP-9 release from human eosinophils and found that the signalling pathway leading to TNF-α-induced MMP-9 release has unique features relative to other granule components from eosinophils and relative to the specific mechanism of TNF-α-induced MMP-9 release from neutrophils (Chakrabarti *et al*., 2006). Wiehler *et al*. suggest that each MAP kinase may be responsible for mediating a unique set of functional responses. Therefore, it will be important to characterize the mechanisms that regulate TNF-α-induced MMP-9 production and release in each cell type. In HIPEC 65 cells, activation of the MAPK pathways by TNF-α induced both phosphorylation of Erk1/2, followed by activation of NF-κB, and phosphorylation of SAPK/JNK, followed by activation of AP-1 (Figure 7). Inhibition of the Erk pathway by treatment with U0126, or inhibition of SAPK/JNK pathway by treatment with SP600 125, resulted in a concomitant decrease of MMP-9 activity, expression and secretion. Interestingly, MMP-2 promoter, which has also an AP-1 site but no NF-κB site (Van den Steen *et al*., 2002), was not activated under these experimental conditions. We have previously shown that AP-1 was clearly implicated in the expression of MMP-9 in primary human CTB (Bischof *et al*., 2003), and we concluded that AP-1 was necessary but probably not sufficient for a complete

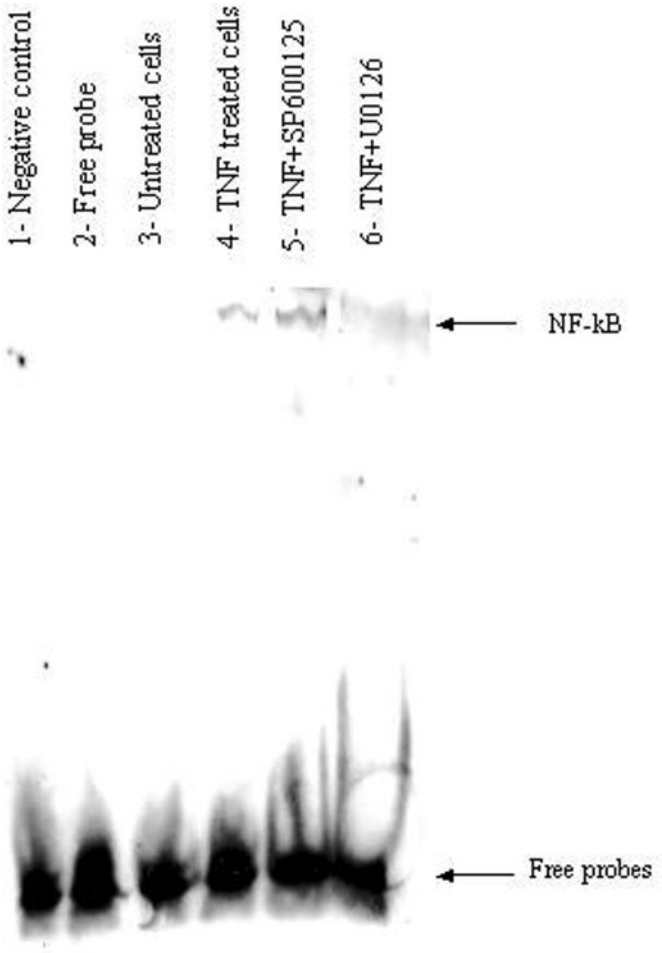

Figure 6. Nuclear factor-κB (NF-κB) DNA-binding capacity in tumour necrosis factor-α (TNF-α)-stimulated HIPEC 65 cells. In the absence of nuclear proteins (lane 2), no chemiluminescence was detected, demonstrating that only biotin-labelled DNA-binding proteins were detected. Unlabeled NF-κB consensus oligonucleotides (lane 1) were added in excess (4 pmoles) for specific competitive assay with biotin-end-labelled NF-κB oligonucleotides. NS, non-specific binding.

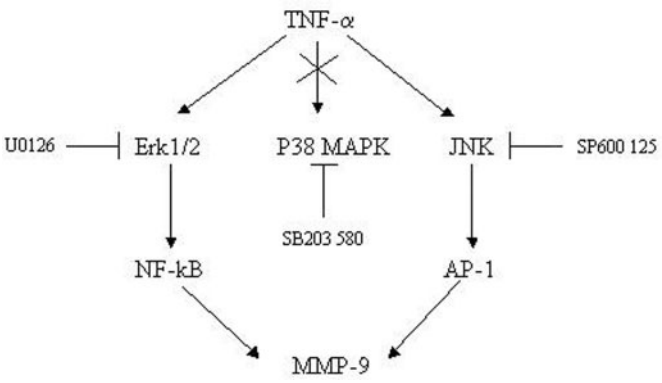

Figure 7. Schematic summary of signal transduction by tumour necrosis factor-α (TNF-α) on metalloproteinase-9 (MMP-9) expression in human trophoblastic cells (HIPEC 65). TNF-α activates different mitogen-activated protein kinase (MAPK) pathways, Erk1/2 and JNK, which in turn initiate nuclear factor-κB (NF-κB) and activator protein 1 (AP-1) activation respectively and finally induce MMP-9 expression. In contrast TNF-α does not activate p38 MAPK in HIPEC 65 cells.

transactivation of MMP-9. We would now propose that NF-κB is also an essential transcription factor in the process of MMP-9 induction by TNF-α in trophoblastic cells. The situation in human trophoblastic cells is not unique, since it is known that TNF-α induced MMP-9 via activation of NF-κB in glioma cells (Esteve *et al*., 2002) and via the activation of NF-κB and AP-1 in osteosarcoma OST cells (Sato and Seiki, 1993).

We also reported here that p38 MAPK is not involved in TNF-α-induced MMP-9 expression in trophoblastic cells, in contrast to other cells (Cho *et al*., 2000; Wiehler *et al*., 2004). Inhibition of the Erk1/2 and SAPK/JNK pathways totally blocked the MMP-9 expression, secretion and activity induced by TNF-α in CTB and HIPEC 65 cells. Thus, for MMP-9 induction by TNF-α in trophoblastic cells, both AP-1 and NF-κB activation seem to be essential, confirming that, acting together, AP-1 and NF-κB synergistically up-regulated gelatinase B, whereas the expression was rather unaffected by the individual transcription factors (Bond *et al*., 2001).

Acknowledgements

The authors express their gratitude to the Swiss National Science Foundation for their financial support and to Pr. Evain-Brion for the donation of HIPEC 65 cell line.

References

Baud V and Karin M (2001) Signal transduction by tumor necrosis factor and its relatives. Trends Cell Biol 11,372–377.

Bauer S, Pollheimer J, Hartmann J, Husslein P, Aplin JD and Knöfler M (2004) Tumor necrosis factor-alpha inhibits trophoblast migration through elevation of plasminogen activator inhibitor-1 in first-trimester villous explant cultures. J Clin Endocrinol Metab 89,2812–2822.

Bischof P, Martelli M, Campana A, Itoh Y, Ogata Y and Nagase H (1995) Importance of matrix metalloproteinases in human trophoblast invasion. Early Pregnancy 1,263–269.

Bischof P, Truong K and Campana A (2003) Regulation of trophoblastic gelatinases by proto-oncogenes. Placenta 24,155–163.

Bond M, Chase AJ, Baker AH and Newby AC (2001) Inhibition of transcription factor NF-kappaB reduces matrix metalloproteinase-1-3, and -9 production by vascular smooth muscle cells. Cardiovasc Res 50,556–565.

Chakrabarti S, Zee JM and Patel KD (2006) Regulation of matrix metalloproteinase-9 (MMP-9) in TNF-stimulated neutrophils: novel pathways for tertiary granule release. J Leukoc Biol 79 (1),214–222.

Chen HL, Yang YP, Hu XL, Yelavarthi KK, Fishback JL and Hunt JS (1991) Tumor necrosis factor alpha mRNA and protein are present in human placental and uterine cells at early and late stages of gestation. Am J Pathol 139,327–335.

Cho A, Graves J and Reidy MA (2000) Mitogen-activated protein kinases mediate matrix metalloproteinase-9 expression in vascular smooth muscle cells. Arterioscler Thromb Vasc Biol 20,2527–2532.

Divya Chhikara P, Mahajan VS, Datta Gupta S and Chauhan SS (2002) Differential activity of cathepsin L in human placenta at two different stages of gestation. Placenta 23,59–64.

Esteve PO, Chicoine E, Robledo O, Aoudjit F, Descoteaux A, Potworowski EF and St-Pierre Y (2002) Protein kinase C-zeta regulates transcription of the matrix metalloproteinase-9 gene induced by IL-1 and TNF-alpha in glioma cells via NF-kappa B. J Biol Chem 277,35150–35155.

Fisher SJ, Leitch MS, Kantor MS, Basbaum CB and Kramer RH (1985) Degradation of extracellular matrix by the trophoblastic cells of first-trimester human placentas. J Cell Biochem 27,31–41.

Fisher SJ, Cui TY, Zhang L, Hartman L, Grahl K, Zhang GY, Tarpey J and Damsky CH (1989) Adhesive and degradative properties of human placental cytotrophoblast cells in vitro. J Cell Biol 109,891–902.

Haynes MK, Jackson LG, Tuan RS, Shepley KJ and Smith JB (1993) Cytokine production in first trimester chorionic villi: detection of mRNAs and protein products in situ. Cell Immunol 151,300–308.

Holvoet S, Vincent C, Schmitt D and Serres M (2003) The inhibition of MAPK pathway is correlated with down-regulation of MMP-9 secretion induced by TNF-alpha in human keratinocytes. Exp Cell Res 290,108–119.

Hunt JS, Chen HL, Hu XL, Chen TY and Morrison DC (1992) Tumor necrosis factor-alpha gene expression in the tissues of normal mice. Cytokine 4,340–346.

King A, Jokhi PP, Smith SK, Sharkey AM and Loke YW (1995) Screening for cytokine mRNA in human villous and extravillous trophoblasts using the reverse-transcriptase polymerase chain reaction (RT-PCR). Cytokine 7,364–371.

Librach CL, Werb Z, Fitzgerald ML, Chiu K, Corwin NM, Esteves RA, Grobelny D, Galardy R, Damsky CH and Fisher SJ (1991) 92-kD type IV collagenase mediates invasion of human cytotrophoblasts. J Cell Biol 113,437–449.

Martelli M, Campana A and Bischof P (1993) Secretion of matrix metalloproteinases by human endometrial cells in vitro. J Reprod Fertil 98,67–76.

Meisser A, Chardonnens D, Campana A and Bischof P (1999) Effects of tumour necrosis factor-alpha, interleukin-1 alpha, macrophage colony stimulating factor and transforming growth factor beta on trophoblastic matrix metalloproteinases. Mol Hum Reprod 5,252–260.

Moon SK, Cha BY and Kim CH (2004) ERK1/2 mediates TNF-alpha-induced matrix metalloproteinase-9 expression in human vascular smooth muscle cells via the regulation of NF-kappaB and AP-1: Involvement of the ras dependent pathway. J Cell Physiol 198,417–427.

Nee LE, McMorrow T, Campbell E, Slattery C and Ryan MP (2004) TNF-alpha and IL-1beta-mediated regulation of MMP-9 and TIMP-1 in renal proximal tubular cells. Kidney Int 66,1376–1386.

Pavan L, Tarrade A, Hermouet A, Delouis C, Titeux M, Vidaud M, Thérond P, Evain-Briond D and Fournier T (2003) Human invasive trophoblasts transformed with simian virus 40 provide a new tool to study the role of PPAR-gamma in cell invasion process. Carcinogenesis 24,1325–1336.

Red-Horse K, Zhou Y, Genbacev O, Prakobphol A, Foulk R, McMaster M and Fisher SJ (2004) Trophoblast differentiation during embryo implantation and formation of the maternal–fetal interface. J Clin Invest 114,744–754.

Renaud SJ, Postovit LM, Macdonald-Goodfellow SK, McDonald GT, Caldwell JD and Graham CH (2005) Activated macrophages inhibit human cytotrophoblast invasiveness in vitro. Biol Reprod 73,237–243.

Sato H and Seiki M (1993) Regulatory mechanism of 92 kDa type IV collagenase gene expression which is associated with invasiveness of tumor cells. Oncogene 8,395–405.

Staun-Ram E, Goldman S, Gabarin D and Shalev E (2004) Expression and importance of matrix metalloproteinase 2 and 9 (MMP-2 and -9) in human trophoblast invasion. Reprod Biol Endocrinol 2,59.

Tovey MG (1989) Expression of the genes of interferons and other cytokines in normal and diseased tissues of man. Experientia 45526–535.

Van den Steen PE, Dubois B, Nelissen I, Rudd PM, Dwek RA and Opdenakker G (2002) Biochemistry and molecular biology of gelatinase B or matrix metalloproteinase-9 (MMP-9). Crit Rev Biochem Mol Biol 37,375–536.

Wajant H, Pfizenmaier K and Scheurich P (2003) Tumor necrosis factor signalling. Cell Death Differ 10,45–65.

Wiehler S, Cuvelier SL, Chakrabarti S and Patel KD (2004) p38 MAP kinase regulates rapid matrix metalloproteinase-9 release from eosinophils. Biochem Biophys Res Commun 315,463–470.

Xu P, Wang YL, Zhu SJ, Luo SY, Piao YS and Zhuang LZ (2000) Expression of matrix metalloproteinase-2, -9, and -14, tissue inhibitors of metalloproteinase-1, and matrix proteins in human placenta during the first trimester. Biol Reprod 62,988–994.

Yang Y, Yelavarthi KK, Chen HL, Pace JL, Terranova PF and Hunt JS (1993) Molecular, biochemical, and functional characteristics of tumor necrosis factor-alpha produced by human placental cytotrophoblastic cells. J Immunol 150,5614–5624.

Submitted on November 29, 2005; accepted on February 2, 2006