Effects of Fibronectin, VEGF and Angiostatin on the Expression of MMPs through Different Signaling Pathways in the JEG-3 Cells

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PROBLEM: The objective of this study was to evaluate the possible signal pathway of fibronectin (FN), vascular endothelial growth factor (VEGF) and angiostatin (AS) on the expression of matrix metalloproteinases (MMPs) in JEG-3 cells.
METHODS OF STUDY: JEG-3 cells were cultured and were examined for the effect of FN, VEGF and AS on the expression of MMPs by immunocytochemistry, gelatin zymography, Western blot analysis and reverse transcription-polymerase chain reaction (RT-PCR).
RESULTS: We found that up-regulation of the expression of MMPs was induced by FN and VEGF through the focal adhesion kinase (FAK)/mitogen-activated protein kinase (MAPK) and Flt-1/p38^{SAPK}/MAPKAPK2 signaling pathways, respectively. Furthermore, AS down-regulated the expression of MMPs through the integrin $\alpha V\beta 3$/FAK signaling pathway independent of the integrin-binding motif Arg-Gly-Asp (RGD).
CONCLUSION: These data indicate that the expression of MMPs is regulated by many independent factors (such as FN, VEGF and AS) through different signaling pathways which influence the behavior of trophoblast cells.

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INTRODUCTION

The implantation of the embryo is a mystery of reproductive biology. There are striking similarities between the embryo implantation and tumor metastasis,[1,2] including angiogenesis and common biochemical mediators, such as the extracellular matrix (ECM), ECM receptors (integrins), ECM degrading enzymes including matrix metalloproteinases (MMPs) and their inhibitors [tissue inhibitors of metalloproteinases (TIMPs)].[3] The development of the placenta requires a series of complex, coordinated interactions between fetal-derived trophoblast cells and the maternal uterus.[4] The invasion of trophoblast cells into the maternal endometrium is one of the key events in human placentation. The process can be viewed as a series of distinct events and is greatly influenced by the factors present in the uterine microenvironment that includes hormones, growth factors and cytokines.[5]

Fibronectin (FN), one of the main components of the ECM, plays a pivotal role in the process of trophoblast cells invasion. The effects of ECM [FN, laminin (LN) and vitronectin (VN)] proteins on the expression of MMP-2 and -9 have been investigated.[6–8] But the signaling pathway through which FN is induced in the trophoblast-like JEG-3 cells, is still unclear.

Vascular endothelial growth factor (VEGF) is a homodimer glycoprotein (34,000–42,000 MW) with potent angiogenic properties because of its mitogenic and migration-stimulating effects on vascular endothelial cells. VEGF has two known receptors: the fms-like tyrosine kinase (Flt)-1 (of which membrane-bound,

as well as several soluble isoforms, are in existence) and the kinase insert domain-containing region (KDR) receptor.[9,10] To date, VEGF has been shown to induce angiogenesis and to be involved in embryo implantation through its receptors in many experimental situations.[11,12]

Angiostatin (AS), a 38,000-MW fragment encompassing the kringle region of plasminogen, has been identified and characterized as a potent inhibitor of neovascularization.[13] It has been reported that AS binds to ATP synthase on the cell surface and that this is related to AS's anti-angiogenic effect (e.g. the down-regulation of endothelial cell proliferation).[14] It has been reported that integrin αVβ3 is a major receptor for AS on bovine arterial endothelial (BAE) cells.[15] Its function and mechanism in the reproductive system, however, is still uncertain.

The invasive ability of trophoblast cells is mediated by matrix degradation enzymes, among which MMPs (such as MMP-2 and -9) play major roles. Both MMP-2 and -9 are able to degrade basement membrane collagen IV (Col IV), FN, LN, elastin, entactin, and proteoglycans as well as gelatin (denatured Col IV). Some studies on the regulation of the expression of MMPs in human trophoblast cells have primarily focused on the role of hormones and cytokines such as hCG and interleukin-1β.[16,17] The effect of FN, VEGF and AS on the expression of MMPs and the signaling pathway involved has not been well elucidated.

In this work, we have analyzed the molecular mechanism whereby FN, AS and VEGF regulate MMPs in JEG-3 cells. We found that the expression of MMPs was up-regulated by FN and VEGF, but is down-regulated by AS through a different signaling pathway.

MATERIALS AND METHODS

Reagents
Polyclonal anti-MMP-2, anti-MMP-9, anti-phospho-p42^{MAPK}, anti-p42^{MAPK}, anti-phospho-FAK, anti-FAK, anti-phospho-MAPKAPK2 and anti-MAPKAPK2 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Monoclonal anti-integrin αVβ3 antibodies were purchased from Santa Cruz Biotechnology too. VEGF, placenta growth factor (PIGF), U0126, SB203580 and echistatin were purchased from Sigma Chemical Co (St Louis, MO, USA). AS was isolated in the Department of Biochemistry and Molecular Biology, the Fourth Military Medical University, China.[18] Antisense FAK ODN (3′-TTT TCT TAC CGT CGA CGA ATA GAA-5′) and sense-FAK oligodeoxynucleotide (ODN) (5′-AAA AGA ATG GCA GCT GCT TAT CTT-3′)[6] were synthesized by the SBS Biological

Company (Beijing, China). Enhanced chemiluminescence (ECL) kits were obtained from Amersham (Arlington Heights, IL, USA).

Cell Line
The choriocarcinoma cell line JEG-3 was provided by Dr Piao Yunshang and Dr Wang Yanling (State Key Laboratory of Reproductive Biology, Institute of Zoology, CAS, Beijing). JEG-3 cells were plated at $1–2 \times 10^5$ cells with 1 mL Dulbecco's modified eagle's medium (DMEM) (Gibco-BRL, Gaithersburg, MD, USA) supplemented with 2 mM sodium pyruvate and 2 mM glutamine in 24-well dishes.[19]

Different Treatments of JEG-3 Cells
Nonstimulated cells as a negative control; FN, cells cultured in 10 μg/mL FN-coated well; AS, cells were treated with 10 μg/mL AS; VEGF, cells were treated with 10 ng/mL VEGF; PIGF, cells were treated with 10 ng/mL PIGF; FN + U0126, cells cultured in 10 μg/mL FN-coated well combined with 20 μM U0126; FN + S-FAK, cells cultured in 10 μg/mL FN-coated well combined with 2 mM sense-FAK ODN; FN + A-FAK, cells cultured in 10 μg/mL FN-coated well combined with 2 mM antisense-FAK ODN; AS + echistatin, cells were treated with 10 μg/mL AS and 10 ng/mL echistatin; AS + A-FAK, cells were treated with 10 μg/mL AS and 2 mM antisense-FAK ODN; AS + S-FAK, cells were treated with 10 μg/mL AS and 2 mM sense-FAK ODN; AS + VEGF, cells were treated with 10 ng/mL VEGF and 10 μg/mL AS; AS + U0126, cells were treated with 10 μg/mL AS and 20 μM U0126; VEGF + SB203580, cells were treated with 10 ng/mL VEGF and 10 mM SB203580; VEGF + anti-Flt-1, cells were treated with 10 ng/mL VEGF and 10 μg/mL anti-Flt-1.

Immunocytochemistry
JEG-3 cells were cultured in chamber slides (Nunc, Roskilde, Denmark). After 24 h in culture, JEG-3 cells were fixed for 30 min in freshly prepared 4% paraformaldehyde (PFA, Sigma) containing 0.2% Triton X-100. After rinsing several times in 0.01 M phosphate-buffered saline (PBS) (pH 7.4), the slides were incubated in 5% bovine serum albumin (BSA) for 45 min at room temperature to block nonspecific binding sites. Then the BSA solution was aspirated with filter paper, and the slides were incubated with the primary antibody diluted 1:500 in PBS at 4°C overnight. After rinsing in PBS, the slides were incubated for 60 min in FITC-conjugated secondary antibody (Sigma), diluted 1:500 in PBS at 37°C, then after a second rinsing in PBS, the slides were incubated for 60 min in propidium iodide (PI) (Sigma). Finally, the slides were viewed under a fluorescent microscope

(Leica, Heidelberg, Germany). Parallel experiments were performed with JEG-3 cells using pre-immune IgG as a negative control.

Gelatin Zymography

The presence of gelatinolytic MMPs in media was detected by gelatin zymography.[20,21] The harvested culture media were standardized according to the protein content of cell lysates, which was measured according to the method of Bradford. Thus, 10–20 µL medium, equivalent to 6 µg protein of cell lysates, was loaded onto each lane for zymography. The medium was mixed 5:1 (v:v) with a sample buffer and then applied to gels for electrophoresis without boiling under nonreducing conditions in 15% acrylamide gel co-polymerized with 1 mg/mL gelatin (Sigma). After electrophoresis, the gels were washed at room temperature for 1 hr in 2.5% Triton X-100, 50 mM Tris–HCl, at pH 7.5, to remove sodium dodecyl sulfate (SDS) and incubated at 37°C for 2–3 days in buffer (150 mM NaCl, 5 mM CaCl$_2$, and 50 mM Tris–HCl, pH 7.6). Thereafter, gels were stained for 60 min with 0.1% (w:v) Coomassie brilliant blue R-250 in 30% (v:v) isopropyl alcohol, 10% glacial acetic acid and destained in 10% (v:v) methanol and, 5% (v:v) glacial acetic acid. Gelatinolytic activities were detected as clear bands on a uniformed blue background. Gels were photographed and the band intensities were quantified by densitometry of the negatives on a protein disulfide isomerase (PDI) protein plus DNA Imageware System (Huntington Station, NY, USA).

Western Blot Analysis

Proteins obtained from cell lysates were boiled in SDS/β-mercaptoethanol sample buffer and about 10 µg proteins were loaded onto each lane of the 12% acrylamide-PAGE gels. The proteins were separated by electrophoresis and the proteins in the gels were blotted onto nylon membrane by electrophoretic transfer in 25 mM Tris, 192 mM glycine buffer, pH 8.3. Blots were blocked in 10% milk for 1 hr. Primary antibodies were diluted 1:500 (to a final concentration of 2 µg/ml IgG) in Tween20/tris-buffered saline (TTBS) (30 mM Tris, pH 7.4, 150 mM NaCl, 0.1% Tween20). After incubation with the primary antibodies overnight at 4°C, the blots were washed 4× 15 min in TTBS, then, incubated for 1 hr in second antibodies (Sigma) diluted 1:5000 in TTBS. The blots were then washed 4× 15 min in TTBS and 2× 15 min in tris-buffered saline (TBS) after which the bands were visualized by enhanced chemiluminescence.

Reverse Transcription-Polymerase Chain Reaction

RNA isolated from JEG-3 cells was subjected to a reverse transcription-polymerase chain reaction (RT-PCR). One microgram of RNA was reverse transcribed (RT) by oligo(dT) priming and Avian myeloblastosis virus (AMV) reverse transcriptase (Promega Corporation, Madison, WI, USA). PCR amplification was carried out on 5 µL of the RT product from JEG-3. PCR cycles were as follows: 95°C, 5 min followed by 35 cycles for cell samples of denaturation (94°C, 30 s), annealing (53°C, 30 s) and extending (72°C, 30 s). Ten microliters of the PCR products were visualized under ultraviolet light on 1.5% agarose gels containing 1µg/mL ethidium bromide (EB). The relative density of the mRNA signals was calculated as the ratios of mRNA signal to β-actin signal. To confirm the amplification of the required cDNA sequence using the PCR primers and conditions described, PCR products were sequenced (data not shown). PCR primers were the following: β-actin upper: 5′-GTG GGG CGC CCC AGG CAC CA-3′, β-actin lower: 5′-CTT CCT TAT TGT CAC GCA CGA TTT C-3′, the expected fragment size is 540 bp. MMP-2 upper: 5′-CAC CTA CAC CAA GAA CTT CC-3′, MMP-2 lower: 5′-AAC ACA GCC TTC TCC TCC TG-3′; the expected fragment size is 332 bp. MMP-9 upper: 5′-TTG AGT CCG GCA GAC AAT CC-3′, MMP-9 lower: 5′-CCT TAT CCA CGC GAA TGA CG-3′, the expected fragment size is 433 bp.[22]

Statistics

Experimental variables were tested in duplicate or triplicate cultures. One-way ANOVA was used for statistical evaluation of data, and significance of the differences between groups was determined by Dunnett's or Scheffe's test as appropriate, with $P < 0.05$ considered significant.

RESULTS

Expression Levels of MMPs and Enzyme Activity were Up-regulated by FN and VEGF, but is Down-regulated by AS

Production of MMP-2 and -9 protein, mRNA and proteolytic activity in JEG-3 cells, in the presence of 10 µg/mL FN, 10 ng/mL VEGF or 10 µg/mL AS, were detected by Western blot, RT-PCR and gelatin zymography respectively. Our results showed that 10 µg/mL FN moderately up-regulated the MMP-2 and -9 mRNA, protein and their proteolytic activity ($P < 0.01$ and $P < 0.01$ respectively). At the same time, the expression levels of MMPs levels and their activity was significantly increased by VEGF ($P < 0.001$; $P < 0.001$); but is dramatically decreased by angiostatin ($P < 0.01$; $P < 0.01$) (Fig. 1).

FN-induced Signal Transduction

Treatment with 10 µg/mL FN stimulated the phosphorylation of focal adhesion kinase (FAK) and

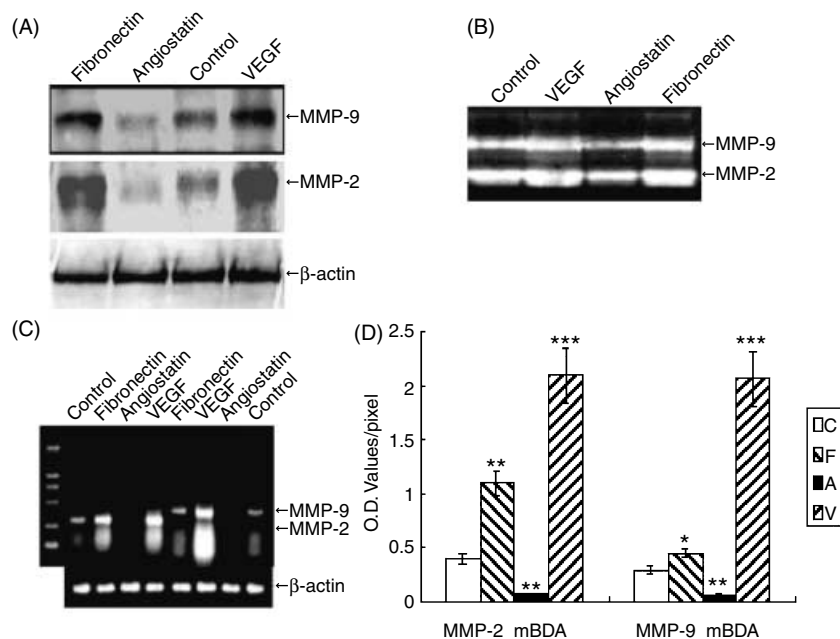*Fig. 1.* Western blot of MMP-2, -9 and β-actin protein expression (A). Gelatin zymography analysis of MMP-2 and -9 (B). RT-PCR products of MMP-2, -9 and β-actin mRNA (C). Densitometric analysis of MMP-2 and -9 mRNA expression (D). 'C' represents control group: DMEM medium without any growth factors; 'A' represents DMEM + 10 μg/mL AS; 'V' represents DMEM + 10 ng/mL VEGF; 'F' represents JEG-3 cells cultured in 10 μg/mL FN-coated well; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.
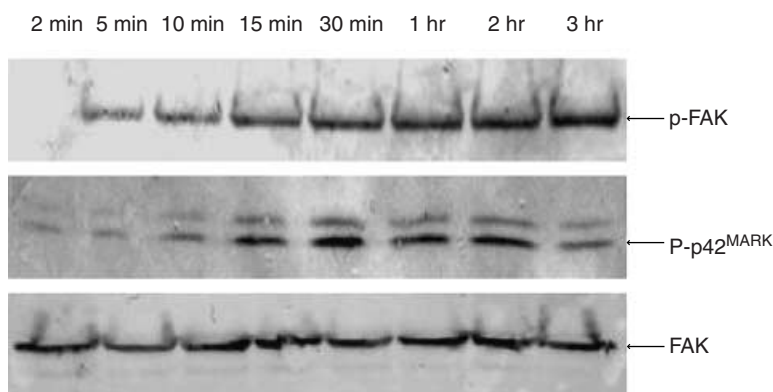

Fig. 2. FN-induced phosphorylation of FAK and p42^{MAPK}. JEG-3 cells were cultured in a 10-μg/mL FN-coated well for various times, and Western blots were prepared from cell lysates. A Western blot was probed with an anti-phospho-FAK (top) or anti-phospho-p42^{MAPK} (middle), and then with anti-FAK (bottom).

p42/p44 mitogen-activated protein kinase (MAPK), extracellular regulating kinase (ERK) in JEG-3 cells (Fig. 2). Interestingly, FN-induced activation of MAPK appeared immediately after treatment, exerting its maximal effects 30 min after treatment. FN-induced activation of FAK appeared after only 5 min of treatment.

Our results showed that FN combined with 20 μM U0126 (specific inhibitor of MEK1) or 2 mM anti-FAK ODN significantly neutralized the effect of FN on MMP-2 mRNA and the activation of p42^{MAPK} ($P < 0.001$). However, sense-FAK ODN had no effect on FN (Fig. 3). Therefore, we speculated that FN-induced the expression of MMPs in JEG-3 cells through the FAK and p42^{MAPK} pathways.

AS-induced Signal Transduction
Treatment with 10 μg/mL AS stimulated the phosphorylation of focal adhesion kinase (FAK) in JEG-3

cells. AS-induced activation of FAK appeared only after 5 min of treatment, exerting its maximal effects at 45 min (Fig. 4).

Production of αVβ3 integrin protein in JEG-3 cells was detected by immunocytochemistry (Fig. 5). As evidenced by confocal immunofluoresence micrography, cells stained with antibodies against αVβ3 integrin displayed intense staining of their membrane and cytoplasm, indicating that these JEG-3 cells had deposits of αVβ3 integrin. The nuclei were stained red by PI. αVβ3 integrin showed green fluorescence. A yellow color represented the overlap of green and red. The negative control showed PI staining only (Fig. 5).

Interestingly, AS combined with 10 μg/mL echistatin or 2 mM anti-FAK ODN significantly neutralized the effect of AS on MMP-2 mRNA expression ($P < 0.01$ or $P < 0.05$) (Fig. 6). But sense-FAK ODN had no effect on AS. Strikingly, Arg-Gly-Asp
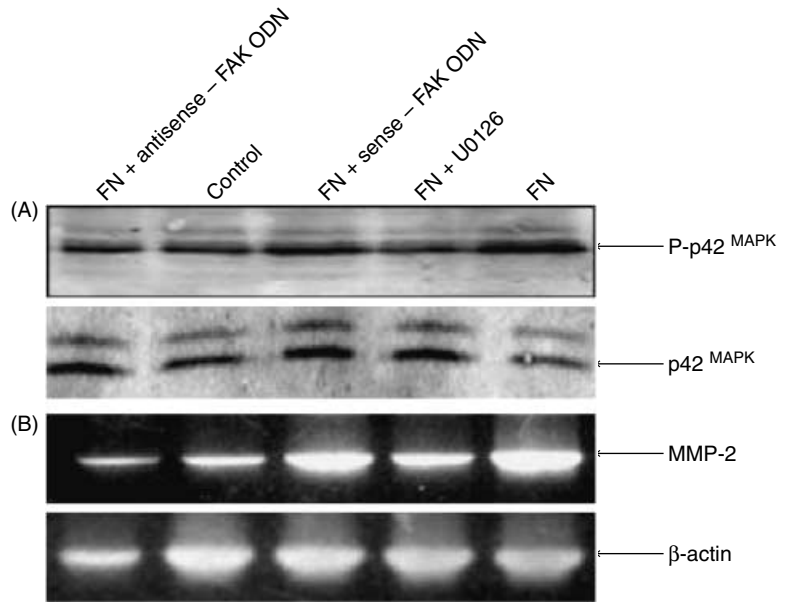
Fig. 3. FN-induced signaling pathway. (A) A Western blot was probed with an anti-phospho-p42MAPK (top) and then with anti-ERK1/2 (bottom). (B) A RT-PCR was probed with MMP-2 primers (top) and then with β-actin primers (bottom). Densitometric analysis of P-MAPK protein and MMP-2 mRNA expression. (C) C, non-stimulated cells; FN, cells cultured in 10 μg/mL FN-coated well; FN + S-FAK, cells cultured in 10 μg/mL FN-coated well combined with 2 mM sense-FAK ODN; FN + A-FAK, cells cultured in 10 μg/mL FN-coated well combined with 2 mM antisense-FAK ODN; FN + U0126, cells cultured in 10 μg/mL FN-coated well combined with 20 μM U0126; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.
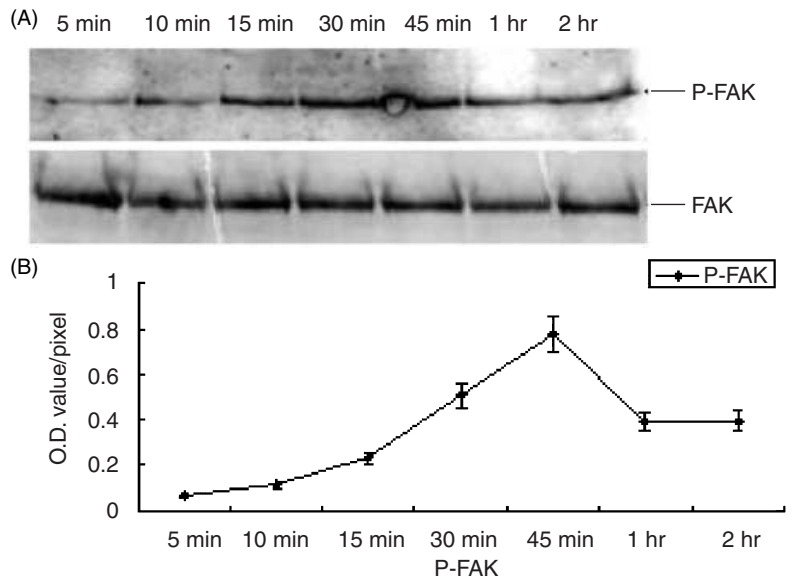
Fig. 4. FAK activation by AS. JEG-3 cells were treated with 10 μg/mL AS for various times, and Western blots were prepared from cell lysates. (A) A Western blot was probed with an anti-phospho-FAK (top) and then with anti-FAK (bottom). Densitometric analysis of P-FAK protein expression (B).
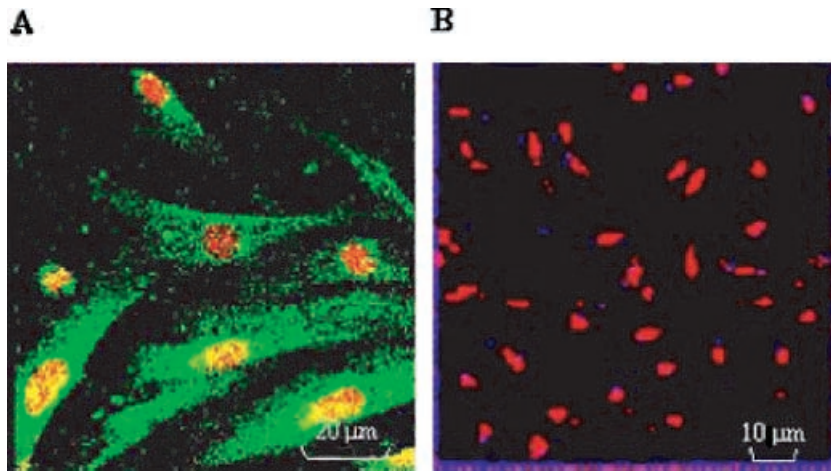
Fig. 5. Expression of αVβ3 integrin in the JEG-3 cells. Slides with JEG-3 were labeled with anti-αVβ3 Integrin, and secondary antibody with anti-rabbit-FITC-conjugated IgG, and then labeled with propidium iodide (PI), as detailed in Materials and Methods [(PI staining of the nucleus (red), immunolabeling of proteins (green)]. The yellow color represents the overlap of green and red. Positive for αVβ3 integrin (A, bar = 20 μm) was observed. Pre-immuno IgG as a negative control (B) (bar = 10 μm).

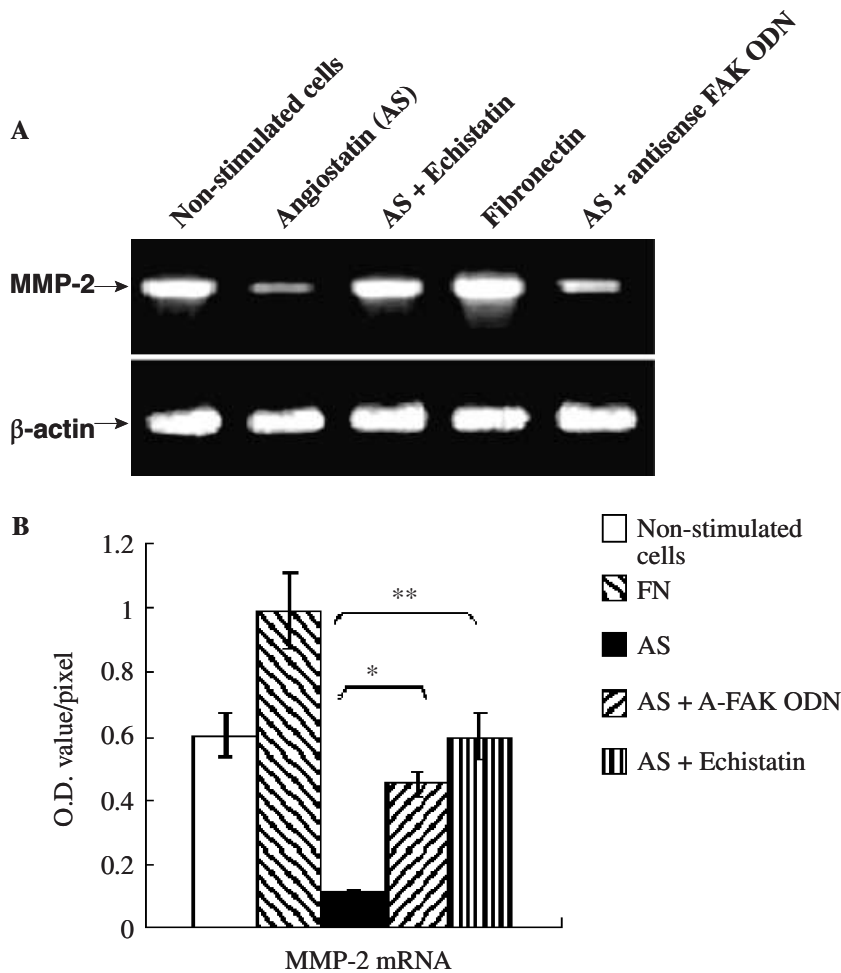

Fig. 6. RT-PCR products of MMP-2 (A, top) and β-actin (A, bottom) mRNA expression. Non-stimulated cells as a negative control; 'AS' represents DMEM + 10 μg/mL AS; 'AS + echistatin' represents DMEM + 10 ng/mL echistatin + 10 μg/mL AS; 'AS + A-FAK ODN' represents DMEM + 10 μg/mL angiostatin + 2 mM antisense-FAK ODN; 'FN' represents JEG-3 cells cultured in 10 μg/mL FN-coated well; densitometric analysis of MMP-2 mRNA expression (B). *$P < 0.05$; **$P < 0.01$.

(RGD) blocked the effect of AS-induced down-regulation of MMP-2 in a dose-dependant manner (Fig. 7). Furthermore, anti-FAK ODN and αVβ3 integrin specific inhibitor, echistatin, significantly decreased the effect of AS on MMP-2 and -9 ($P < 0.05$; $P < 0.05$; $P < 0.01$; $P < 0.001$) (Figs 8 and 9), but MEK1 specific inhibitor, U0126, and sense-FAK ODN had no effect on AS (Figs 8 and 9). Therefore, we speculated that the AS-induced down-regulation of MMPs through the αVβ3 integrin/FAK pathway and RGD is a kind of antagonist of AS.

A

B

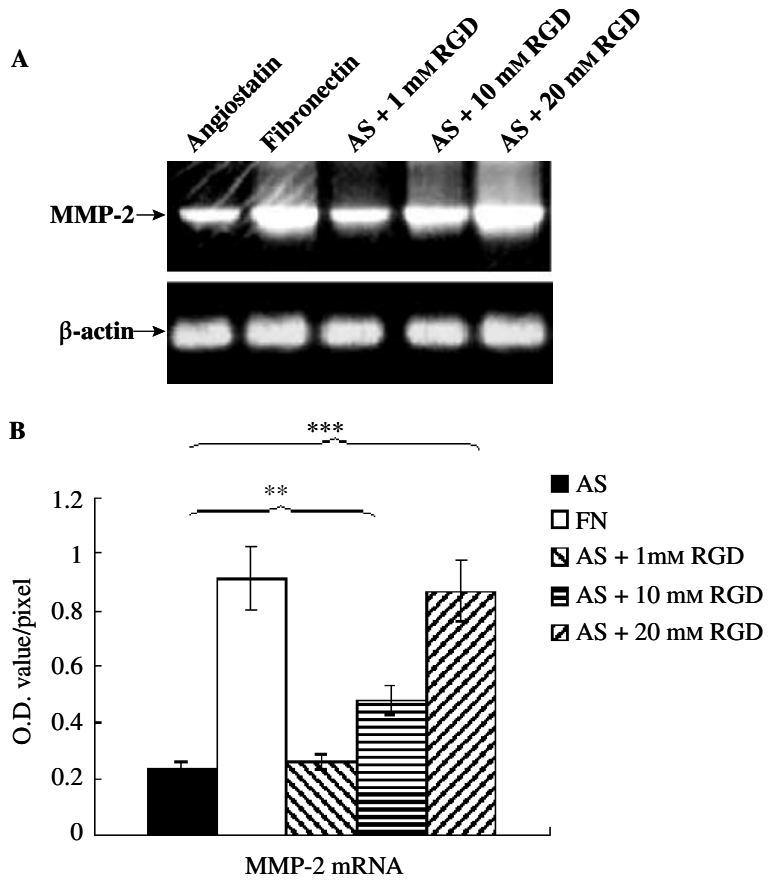*Fig. 7.* RGD is a kind of antagonist of angiostatin in JEG-3 cells. (A) RT-PCR product of MMP-2 mRNA (top) and β-actin mRNA (bottom). 'AS' represents 10 μg/mL AS; 'FN' represents JEG-3 cultured in a 10 μg/mL FN precoated well; AS + 1 mM RGD, 10 μg/mL AS plus 1 mM RGD; AS + 10 mM RGD, 10 μg/mL AS plus 10 mM RGD; AS + 20 mM RGD, 10 μg/mL AS plus 20 mM RGD. Densitometric analysis of MMP-2 mRNA expression (B). **$P < 0.01$; ***$P < 0.001$.

VEGF-induced Signal Transduction is Affected by AS
Production of KDR and Flt-1 protein in JEG-3 cells was detected by immunocytochemistry (Fig. 10). As evidenced by confocal immunofluoresence micrography, cells stained with antibodies against Flt-1 had intense staining of their cytoplasm, indicating that these JEG-3 cells had cellular deposits of Flt1 (Fig. 10A). The nuclei were stained red by the PI, Flt-1 showed green fluorescence. A yellow color represented the overlap of green and red. But there is no KDR production in JEG-3 cells (Fig. 10B).

PlGF has been demonstrated to bind Flt-1 with high affinity, but not KDR/Flk-1.[20] In our study, treatment with 10 ng/mL PlGF or 10 ng/mL VEGF both up-regulated MMP-2 mRNA expression (Fig. 11). Therefore, we speculated that there is Flt-1 but no KDR expression in the JEG-3 cells.

Treatment with 10 ng/mL VEGF stimulates the phosphorylation of p38[SAPK] and MAPKAPK2 (Fig. 12), which is a down-stream kinase of p38[SAPK]. In addition, anti-Flt-1 and p38[SAPK] specific inhibitor, SB203580, dramatically decreased the effect of VEGF on MMP-9 ($P < 0.001$) (Fig. 9). Thus we speculated that VEGF-induced the up-

regulation of MMPs through the Flt-1/MAPKAPK2 pathway.

Interestingly, AS down-regulated protein expression of VEGF and Flt-1 in a dose-dependent manner in JEG-3 cells (Fig. 13). Furthermore, AS significantly diminished VEGF induced the activation of p38[SAPK] and MAPKAPK2 ($P < 0.001$ and $P < 0.001$) (Fig. 14).

DISCUSSION

Human implantation and subsequent placental development requires a series of complex and coordinated interactions between the fetal-derived trophoblast cells and the maternal uterus. As the trophoblast cells migrate through the decidual stroma, they are confronted with various basement membranes and matrix substrates. The invasive ability of trophoblast cells is mediated by matrix degradation enzymes, such as MMPs. MMP-2 and -9 play important roles in this process. Many factors (such as steroid hormones and cytokines) are involved in the regulation of the expression of MMPs.[20,24–27] In our current study, we found that FN and VEGF up-regulated the expression

A

B
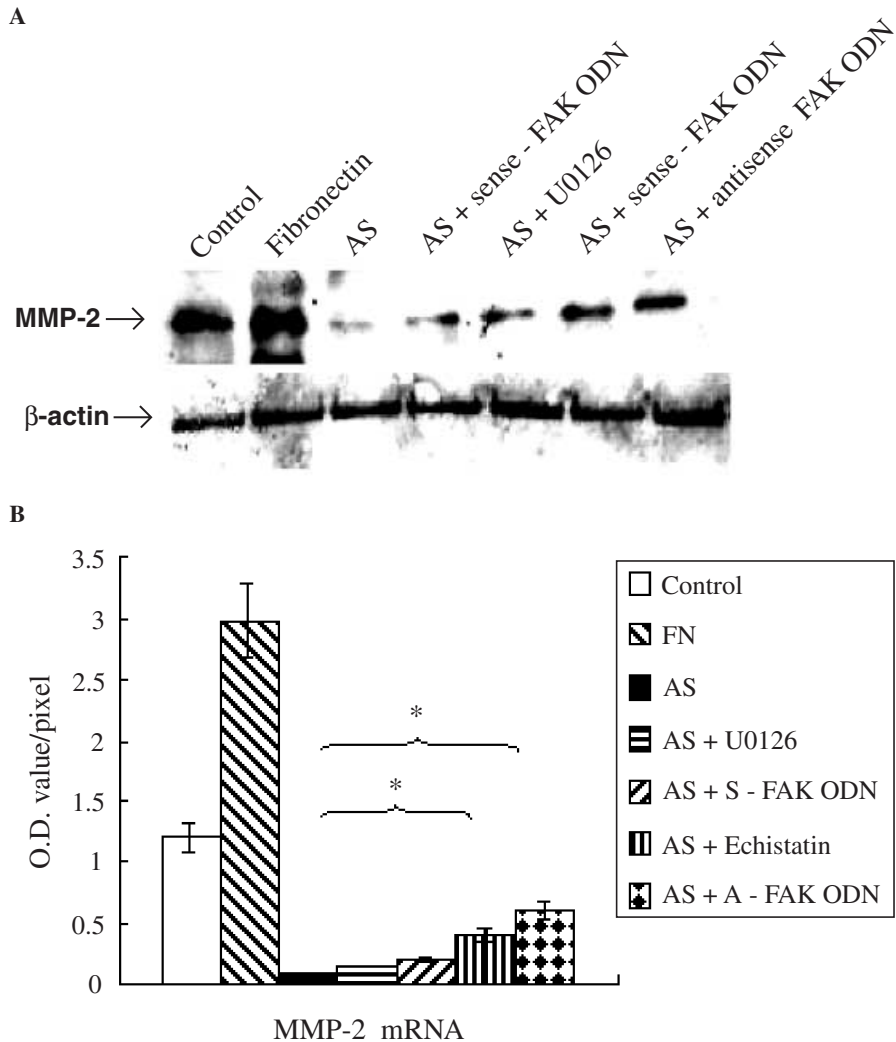
Fig. 8. Effect of AS on MMP-2 protein expression by AS-induced signaling pathway. (A) A Western blot was probed with anti-MMP-2 (top) and then with β-actin (bottom). (B) Densitometric analysis of MMP-2 mRNA expression. DMEM medium without any growth factors as control group; FN (JEG-3 cells cultured in a 10 μg/mL FN precoated well), AS (10 μg/mL AS), AS + U0126 (10 μg/mL AS + 10 mM U0126), AS + sense-FAK ODN (10 μg/mL AS + 2 mM sense-FAK ODN), AS + Echistatin (10 μg/mL AS + 10 ng/mL echistatin) and AS + antisense-FAK ODN (10 μg/mL AS + 2 mM antisense FAK ODN) treatment respectively. *$P < 0.05$.

of MMP-2 and -9, whereas AS down-regulated the expression of MMPs in the trophoblast-like JEG-3 cells.

AS, the N-terminal four kringles (K1–4) of plasminogen, blocks tumor-mediated angiogenesis and has great therapeutic potential. It has been shown that BAE cells adhere to AS in an integrin-dependant manner and that integrins αVβ3, α9β 1, and to a lesser extent α4β1, specifically binds to AS. Integrin αVβ3 have been shown to be a predominant receptor for AS on BAE cells. In the current study, we found that integrin αVβ3 might be a receptor of AS in JEG-3 cells, as a function-blocking inhibitor of integrin αVβ3 (i.e. echistatin) effectively blocks the effect of AS on the expression of MMPs.

The interaction of cells with ECM proteins generates intracellular signals important for the growth, survival, and migration of cells. The integrin family of transmembrane receptors has long been recognized for their structural roles in linking ECM proteins with the cellular actin cytoskeleton in the regulation of cell shape and tissue architecture. Recent developments within the field of integrin biology have shown that integrin receptors can initiate signal transduction events that affect many aspects of cell growth.[28–30]

The FAK protein is a nonreceptor and nonmembrane associated PTK which does not contain Src homology 2 (SH2) or SH3 protein interaction domains.[30] FAK and its role in linking integrin receptors to intracellular signaling pathways play a
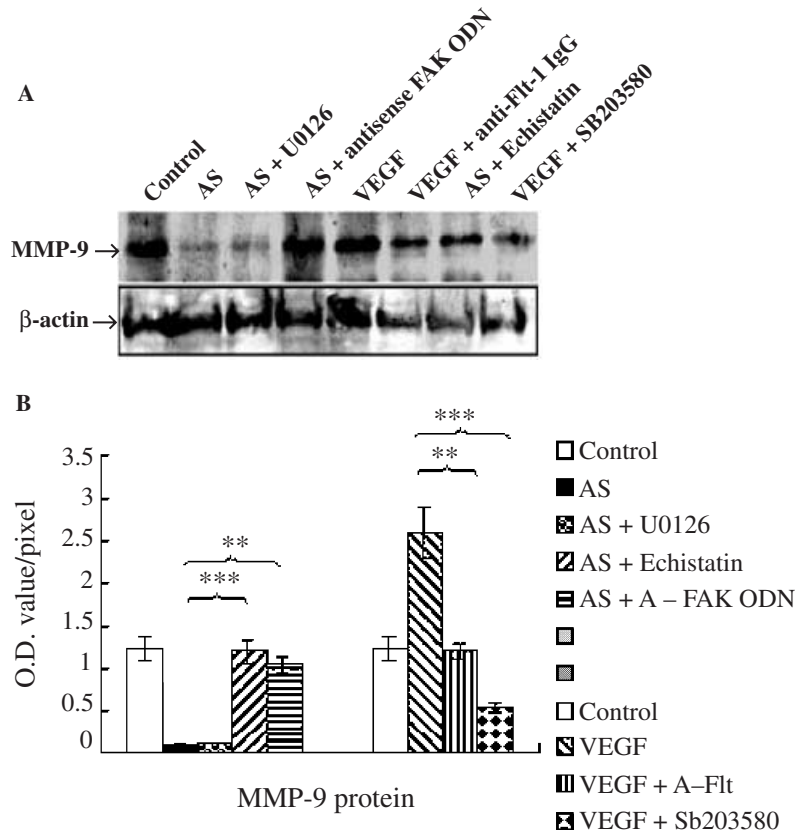
Fig. 9. Effect of AS and VEGF on MMP-9 protein expression via different signaling pathways. (A) A Western blot was probed with anti-MMP-9 (top) and then with β-actin (bottom). Densitometric analysis of MMP-9 protein expression (B). C (DMEM medium without any growth factors), AS (10 μg/mL AS), AS + U0126 (10 μg/mL AS + 10 mM U0126), AS + anti-FAK ODN (10 μg/mL AS + 2 mM antisense FAK ODN), VEGF (10 ng/mL VEGF), VEGF + anti-Flt-1 (10 ng/mL VEGF + 10 μg/mL anti-Flt-1 IgG), AS + Echistatin (10 μg/mL AS + 10 ng/mL echistatin) and VEGF + SB203580 (10 ng/mL VEGF + 10 mM SB203580) treatment respectively. $**P < 0.01$; $***P < 0.001$.

pivotal role in cells. In this study, the FAK signaling pathway was found to play two different roles in the effect of AS and FN. On the one hand, FN, one of the ECM proteins, binding to its receptor integrin, activates the phosphorylation of FAK. This enables FAK to function within a network of integrin-stimulated signaling pathways leading to the activation of targets such as the ERK/MAPK pathways. Ultimately, the expression of the MMPs gene is up-regulated by FN through the FAK/MAPK signaling pathway. On the
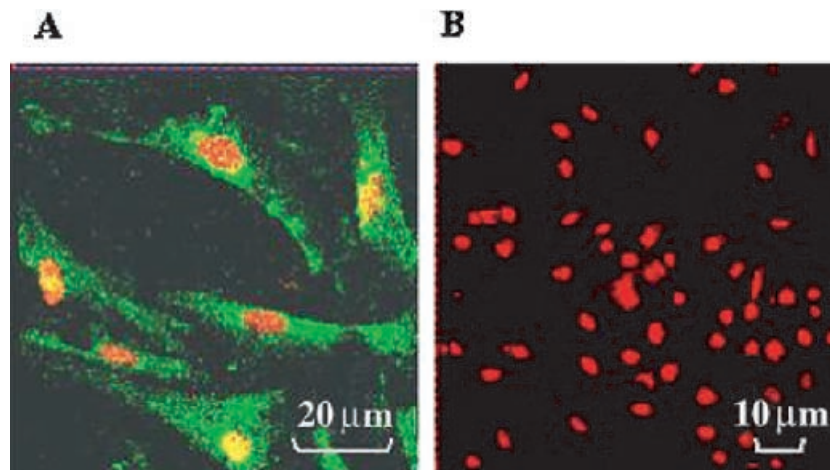
Fig. 10. Expression of Flt-1, but not KDR in the JEG-3 cells. Slides with JEG-3 were labeled with anti-Flt-1 or KDR, secondary antibody anti-rabbit-FITC-conjugated IgG, and then with PI respectively, as detailed in Materials and Methods [PI staining of the nucleus (red), immunolabeling of matrix proteins (green)]. The yellow color represents the overlap of green and red. A positive reaction for Flt-1 (A, bar = 20 μm; B, bar = 50 μm) and a negative reaction for KDR (B) were observed (bar = 10 μm).

A

B
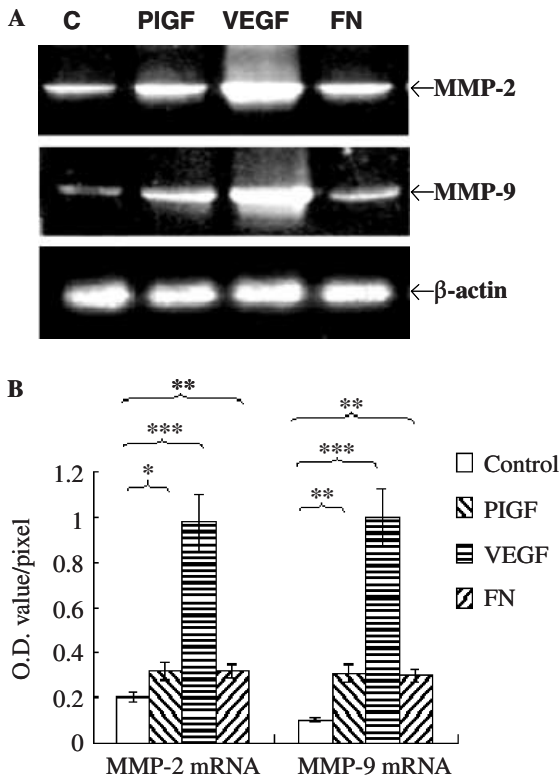
Fig. 11. RT-PCR products of MMP-2 (A, top) and MMP-9 (A, middle) mRNA expression. 'C' represents control group: DMEM medium without any growth factors. 'PlGF' represents DMEM + 10 ng/mL PlGF. 'VEGF' represents DMEM + 10 ng/mL VEGF. 'FN' represents JEG-3 cells cultured in 10 μg/mL FN-coated well. Densitometric analysis of MMP-2 and -9 mRNA expression (B). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.
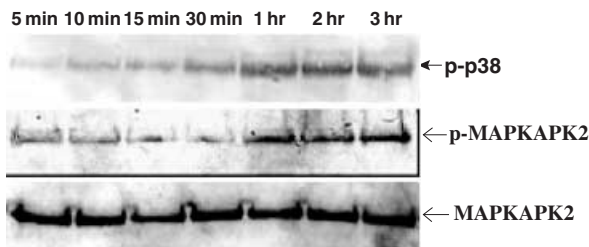
Fig. 12. Temporal-dependent p38 and MAPKAPK2 activation in response to VEGF in JEG-3 cells. Cell lysates were prepared from JEG-3 cells after treatment with 10 ng/mL VEGF. A Western blot was probed with anti-phospho-p38 (top), anti-phospho-MAPKAPK2 (middle) and then with anti-total MPKAPK2 (bottom).

other hand, AS binding to its receptor, integrin αVβ3, activated the phosphorylation of FAK and down-regulated the expression of MMPs in JEG-3 cells. Interestingly, as treatment with the AS-neutralizing RGD counteracted this effect, AS induced the expression of MMPs and activation of FAK
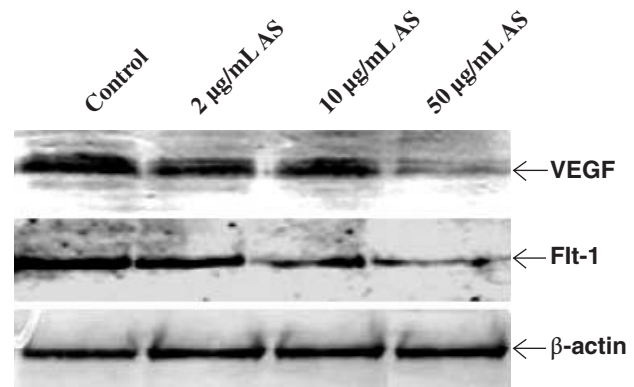
Fig. 13. VEGF and Flt-1 is down-regulated by AS. A Western blot was probed with an anti-VEGF (top) or anti-Flt-1 (middle) and then with anti-β -actin (bottom). Nonstimulated cells as a negative control.

independent of the integrin-binding motif RGD in JEG-3 cells. Therefore, we speculate that RGD acts as an antagonist of AS, binding to integrin αVβ3 in the JEG-3 cells.

There is a strikingly similarity between the endothelial and trophoblast cells. Endothelial cells degrade the basal lamina and invade the underlying interstitial space, where they proliferate and eventually regroup to form patent capillaries. Trophoblast cells degrade ECM and invade the uterine endometrium, where they proliferate and eventually connect tightly with the maternal uterus. Both VEGF and AS are involved in angiogenesis with endothelial cells as the central players. On the other hand, VEGF is one of the angiogenic mitogens which stimulate endothelial cell proliferation,[31] whereas, AS is one of the antiangiogenic factors which inhibit endothelial cell proliferation. It has been reported that AS reduced the phosphorylation of the MAPKs ERK-1 and -2 transiently in human dermal microvascular cells, but not in human vascular smooth muscle cells or human dermal fibroblasts.[32] In this study, we detected the effect of VEGF and AS in the trophoblast-like cell, JEG-3.

To date, five VEGF splice variants of 121, 145, 165, 189, and 206 amino acids have been identified in human tissues. Two laboratories reported that VEGF$_{165}$ is capable of binding to both Flt-1 and Flk-1. Flt-1 but not KDR tyrosine kinase is a receptor for the PlGF in the trophoblast cells.[23] In our study, treatment with 10 ng/mL PlGF up-regulated MMP-2 mRNA expression (Fig. 11). Furthermore, immunocytochemistry revealed that JEG-3 cells had cellular deposits of Flt-1, but not KDR, suggesting that there is only Flt-1 (but no
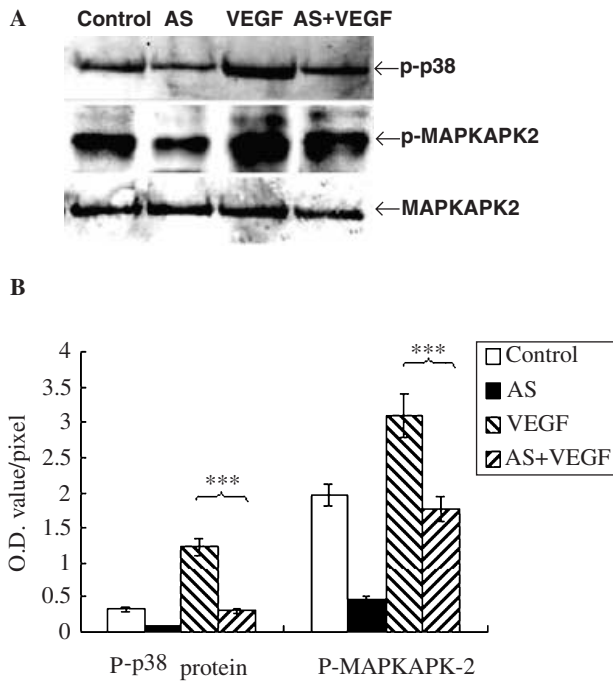
A

B

Fig. 14. Angiostatin blocked the VEGF induced p38 and MAP-KAPK2 signaling pathway in JEG-3 cells. (A) A Western blot was probed with anti phospho-p38 (top) and anti-phospho-MAPKAPK2 (middle) and then with anti-MAPKAPK2 (bottom). Densitometric analysis of P-p38 and P-MAPKAPK-2 protein expression (B). Non-stimulated cells as a negative control; 'AS' represents 10 μg/mL angiostatin; 'VEGF' represents 10 ng/mL VEGF and 'AS + VEGF' represents 10 μg/mL angiostatin + 10 ng/mL VEGF. ***$P < 0.001$.

KDR) expression in JEG-3 cells. Unlike the effect of $VEGF_{165}$ in endothelial cells, $VEGF_{165}$ binding to its Flt-1 but not KDR/Flk-1 receptor up-regulated the expression of MMPs through the $p38^{SAPK}$/MPKAPK2 signaling pathway, leading to MMPs degrading the ECM substrate. U0126 had no effect on $VEGF_{165}$ in this study (data not shown). Further-

more, AS blocked the activation of MAPKAPK2, the substrate of $p38^{SAPK}$, and the up-regulation of the expression of MMPs by VEGF.

A variety of factors have been identified that regulate the expression of MMPs. Many of these factors have already been shown to influence key functions of human trophoblast cells. For example, leukaemia inhibitory factor (LIF), tumor necrosis factor (TNF), transforming growth factor beta (TGF-β), interleukin-1 and -6 (IL-1, IL-6) and insulin-like growth factor binding protein (IGFBP-1) as well as trophoblastic factors such as hCG and leptin have been found to markedly influenced the secretion and/or activation of MMP-2 and -9. Most cytokines influence cell behavior by modulating the phosphorylation of transcription factors. Among these transcription factors two oncogene products (Jun and Fos) have been found to be activated by TNF or phorbol ester and promote the synthesis of MMP-9.[33] In this study, we added VEGF and AS to this list. Taken together, these findings reveal that the expression of MMPs and the behavior of trophoblast cells are regulated by many independent factors, that is, FN binding to integrin regulates MMP expression through the FAK/p42^{MAPK} signaling pathway. VEGF binding to its receptor Flt-1 but not KDR regulates the expression of MMPs through the $p38^{SAPK}$/MAPKAPK2 signaling pathway. AS binding to integrin αVβ3 regulates the expression of MMPs through the FAK signaling pathway independent of the integrin-binfing motif RGD. AS blocked activation of $p38^{SAPK}$ and MAPKAPK2 by VEGF (Fig. 15).

Acknowledgements

Fig. 15. Proposed intracellular signaling actions of FN, VEGF and AS on MMP expression in JEG-3 cells. FN binding to integrin regulates MMPs expression through the FAK/p42^{MAPK} signaling pathway. VEGF binding to its receptor Flt-1 but not KDR regulates the expression of MMPs through the $p38^{SAPK}$/MAPKAPK2 signaling pathway. AS binding to integrin αVβ3 regulates the expression of MMPs through the FAK singling pathway independent of the integrin-binding motif RGD. AS blocked activation of $p38^{SAPK}$ and MAP-KAPK2 by VEGF.
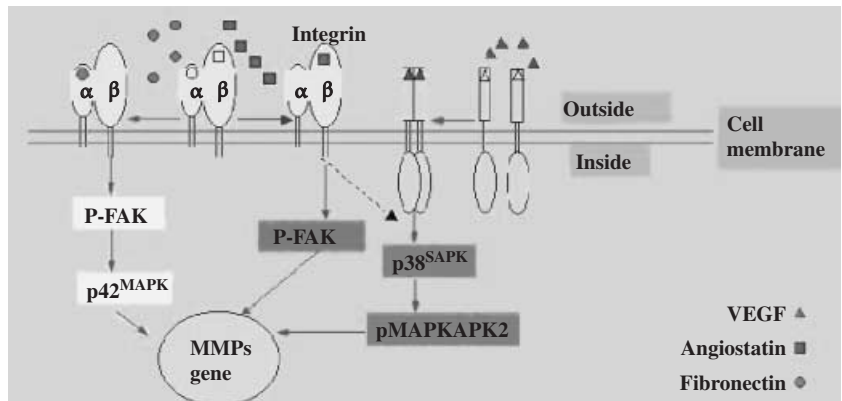
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