Modulation of Matrix Metalloproteinase Secretion by Adenosine A3 Receptor in Preeclamptic Villous Explants

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Modulation of Matrix Metalloproteinase Secretion by Adenosine A3 Receptor in Preeclamptic Villous Explants

Directed by Professor Yong-Won Park

The Doctoral Dissertation
submitted to the Department of Medicine,
the Graduate School of Yonsei University
in partial fulfillment of the requirements for the degree
of Doctor of Philosophy

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December 2007

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December 2007

감사의 글

산부인과학 전공의 과정을 마치고 모성태아의학에 입문한 지 어언 8년 여가 흘렀습니다. 힘들었고 많이 부족했던 연구를 이제 마무리하고 다시 새로운 발걸음을 옮기려 합니다. 그 동안 저를 옆에서 지켜 보며 힘을 주셨던 모든 분들께 감사의 마음을 전하려 합니다.

오래 전부터 제 인생의 mentor가 되어 주셨고 본 연구에 지원과 관심을 아끼지 않으신 박용원 지도 교수님께 깊이 감사드립니다. 항상 저에게 자상한 가르침을 주셨던 김영태 교수님, 김행수 교수님께도 감사드립니다. 연구의 부족한 점에 대해 날카로운 지적을 아끼지 않으신 김정훈 교수님께 감사드립니다. 그리고 항상 바쁘신 와중에도 제 연구에 많은 관심을 보여주셨던 윤주헌 교수님께도 감사드립니다.

5년 전 떠났던 미국 연수 기간 동안 저에게 많은 힘을 주셨고 본 연구에 결정적인 영감을 주신 Brian J. Koos 교수께 감사드립니다. 본 연구의 실험 진행에 많은 도움을 준 후배 황한성 강사, 그리고 생화학교실의 맹용선 연구원에게도 깊은 감사의 마음을 전합니다.

제 인생의 항상 든든한 버팀목이 되어 주시는 사랑하는 부모님, 아들처럼 저를 아껴주시는 장인, 장모님께 감사드립니다. 힘들때면 언제나 저보다 훨씬 더 강한 정신력으로 남편을 항상 격려해주고 지금의 저를 있게 해 준 사랑하는 아내 정완에게 감사의 마음을 전합니다. 밤늦게 들어와 잘 놀아주지도 못하지만 항상 못난 아빠를 반겨주는 사랑하는 딸 건아, 아들 건우에게도 고마움을 느끼며 앞으로 더 많은 사랑을 주려 합니다.

모두들 감사드립니다.

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<ABSTRACT>

Modulation of Matrix Metalloproteinase Secretion by Adenosine A3 Receptor in Preeclamptic Villous Explants

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Objective: Adenosine, known to be released from inflammatory sites and tissue ischemia, has many important biologic roles. Four specific adenosine receptors have been cloned to date, termed A1, A2a, A2b, and A3 receptors. Recently our study has shown that A3 receptor in the trophoblast of preeclamptic pregnancy was increased, suggesting that non-vascular and trophoblast-mediated A3 receptor may play an important role in the pathogenesis of preeclampsia. There are evidences of impaired trophoblast invasion related to matrix metalloproteinase (MMP) in preeclampsia and the relationship between adenosine receptor and MMP in other fields. The objective of this study is to evaluate modulation of MMP secretion by adenosine A3 receptor in preeclamptic villous explants at different oxygen conditions.

Methods: Placental villous explants from normal (n=10) and preeclamptic (n=10) pregnancies were cultured at high (20%) and low (3%) oxygen levels for 5 days. The expression levels of MMP-2/-9 and tissue inhibitor of metalloproteinase (TIMP)-1/-2 were analyzed in the

explants by RT-PCR and Western blot. Thereafter, preeclamptic villous explants in hypoxic culture condition were treated with A3 receptor agonist, Cl-IB-MECA and A3 receptor antagonist, MRE. And then MMP-2/-9 expression was determined in a time- and dose-dependent manner by RT-PCR, western blot. Also MMP-2/-9 activity was evaluated by zymogram assay.

Results: There were significantly increased A3 receptor intensity and reduced MMP-2/-9 and TIMP-1/-2 expression at low oxygen level in normal and preeclamptic villous explants. Interestingly, in preeclamptic villous explants, after high oxygen culture for 5 days, the expression of MMP-2/-9 and TIMP-1/-2 expression were recovered to almost same level of those in normal villous explants. Treatment of preeclamptic villous explants with A3 receptor agonist, Cl-IB-MECA in low oxygen level resulted in an enhanced expression of MMP-2 and MMP-9 in a time- and dose-dependent manner. This Cl-IB-MECA-induced expression of MMP-2 and MMP-9 was inhibited by pretreatment with A3 receptor antagonist, MRE.

Conclusion: To the best of our knowledge, this is the first study to evaluate the modulation of MMP secretion by adenosine A3 receptor in preeclamptic villous explant. Our results provide evidence for the existence of functional adenosine A3 receptor in the trophoblast and suggest that adenosine A3 receptor could be used as a therapeutic target in preeclampsia with further investigation.

Key words : adenosine A3 receptor, matrix metalloproteinase, preeclampsia, villous explants

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I. INTRODUCTION

The nucleoside adenosine is released from activated or stressed cells and dramatically accumulates in tissues during ischemia, inflammation, and tissue damage.[1] This ubiquitous molecule is used in selective signaling, activating membrane-bound adenosine receptors, which are widely distributed in tissues and mediate diverse biologic effects.[2] Adenosine exerts its actions by binding to specific, high affinity G-protein coupled receptors. Four specific adenosine receptors have been cloned to date, termed A1, A2a, A2b, and A3 receptors.[3] Adenosine has been recognized as an important modulator of immune responses, mediating inflammatory as well as anti-inflammatory effects, such as chemotaxis,[4-6] release of allergic mediators,[7] reduction of toxic oxygen

metabolites,[8] neutrophil adherence to endothelium,[9] inhibition of T-cell activation and cytokine production.[10-13]

Preeclampsia is a disease affecting about 5% of pregnancies and is clinically diagnosed by the onset of hypertension and proteinuria.[14] Although the etiology of this disease is uncertain, it has been widely accepted that a defect in placental trophoblast invasion during implantation contributes to inadequate remodeling of uterine spiral arteries, thereby initiating focal regions of reduced perfusion within the placenta.[15] This invasion of cytotrophoblasts is partly regulated by the secretion of proteases, in particular matrix-metalloproteinases.[16]

Matrix metalloproteinases (MMPs) are a family of calcium-dependent, zinc-containing endopeptidases that are structurally and functionally related.[17] They are secreted in an inactive (latent) form, which is called a zymogen or a pro-MMP. These latent MMPs require an activation step before they are able to cleave extracellular matrix (ECM) components. The activity of MMPs is regulated by several types of inhibitors, of which the tissue inhibitors of metalloproteinases (TIMPs) are the most important.[18] The TIMPs are also secreted proteins, but they may be located at the cell surface in association with membrane-bound MMPs. The balance between MMPs and TIMPs is largely responsible for the control of degradation of ECM proteins. MMPs are involved in the remodeling of tissues during embryonic development, cell migration, wound healing, and tooth development.[19-22] However, a deregulation of the balance between MMPs and TIMPs is a characteristic of diverse pathological conditions, such as rheumatoid and osteoarthritis, cancer progression, and acute and chronic cardiovascular diseases.[17,18, 23, 24]

It has been demonstrated that human trophoblast invasiveness in vitro depends on the production of MMPs and that both MMP-2 and MMP-9 are secreted by human trophoblasts isolated from first trimester placenta.[25] Previous studies indicate that both mRNA and protein of MMP-2 are found in

cytotrophoblasts from cell columns of anchoring villi as well as decidual cells of first trimester human pregnancies, and trophoblast from third trimester placenta secretes primarily MMP-9 and minimal amounts of MMP-2.[26-28] Also, placental explants from IUGR pregnancies demonstrated reduced MMP-2, MMP-9, and TIMP-1 release compared with explants from normal pregnancies at high (20%) but not low (3%) oxygen.[29]

Recent two studies have demonstrated that there is a relationship between adenosine receptor and MMP. The addition of the adenosine A1 agonist CHA stimulated the secretion of MMP-2 from trabecular meshwork cells.[30] These result provided evidence for the existence of functional adenosine receptors in the trabecular cells and that the activation of these receptors stimulates secretion of MMP-2. Also, adenosine inhibited MMP-9 secretion by neutrophils via A2a receptor.[31]

Our previous study has shown that the subtypes of adenosine receptors were differentially expressed in the human placenta of preeclamptic pregnancy.[32] Western blotting revealed that A2a, A2b, and A3 receptors were all present in the placental tissue. The bands for those receptors were significantly stronger in preeclamptic placenta compared to that of the normal placenta. A2a and A2b receptors were detected in endothelial cells, whereas A3 receptors were absent. Modest staining of A2a and A2b receptors in endothelial cell membrane were showed in preeclamptic placental tissue when compared to the normal placental tissue. Importantly, A3 antibody stained with higher intensity in cyto- and syncytiotrophoblast in preeclamptic placental tissues. This study suggests that non-vascular and trophoblast-mediated A3 receptor may play an important role in the pathogenesis of preeclampsia.

Thus, the objective of this study was to evaluate the modulation of MMP secretion by adenosine A3 receptor in normal and preeclamptic villous explants at both high and low oxygen levels.

II. MATERIALS AND METHODS

1. Reagents

Solutions of adenosine A3 receptor agonist, 2-chloro-*N*6-3-iodobenzyladenosine-5'-*N*-methyluronamide (Cl-IB-MECA) (Sigma-Aldrich, Steinheim, Germany) and adenosine A3 receptor antagonist, 5*N*-(4-methoxyphenylcarbamoyl)amino-8-propyl-2-(2-furyl)-pyrazolo[4,3-e][1, 2,4]triazolo[1,5-c]pyrimidine (MRE3008F20) (Sigma-Aldrich, Steinheim, Germany), were dissolved in deionized water just before the use of agent.

2. Placental villous explants culture

Human term placentas were obtained after vaginal or cesarean deliveries from normal (n=10) and preeclamptic pregnancies (n=10) under the approval of the institutional review board. Three to five cotelydons were extracted at random and rinsed extensively with sterile saline. Decidual tissues and large vessels were removed from villous placenta by blunt dissection. The villous tissue was then finely dissected into 5 mg pieces in sterile PBS and antibiotics (penicillin, streptomycin, and gentamicin) to remove maternal blood. The pieces of tissues were then washed twice in the above solution, and 5 pieces (30–50 mg tissue) were placed into 24-well plates containing 1ml phenol-free medium 199 (Life Technologies, Gaithersburg, MD) supplemented with 2% Nutridoma HS (Boehringer Mannheim, Indianapolis, IN) and antibiotics. Explants were incubated at 37^{o}C for 5 days at 20% oxygen (to simulate high oxygen conditions) and at 3% oxygen (to simulate low oxygen conditions).

The diagnosis of severe preeclampsia was based on the definitions set by the American College of Obstetricians and Gynecologists.[33] Severe preeclampsia

was defined as the presence of hypertension and proteinuria after the 20th week of pregnancy; blood pressure elevation with a systolic blood pressure of 160 mm Hg or higher or a diastolic blood pressure of 110 mm Hg or higher; and proteinuria greater than 1000 mg per 24 h or a reading of at least 3+ on dipstick was considered significant. At least 2 consecutive positive measurements on urinalysis were required for diagnosis.

3. Semi-quantitative RT-PCR Analysis (sqRT-PCR)

Total RNA was obtained from villous tissues with a TRIzol reagent kit (Invitrogen, Carlsbad, CA). 0.5–5 μg RNA samples were used in the reverse transcriptase-polymerase chain reactions (RT-PCR), and the correlation between the amounts of RNA used and quantity of PCR products from MMPs and TIMPs mRNA and the internal standard (GAPDH) mRNA was examined. The primers used are given in table 1. Briefly, target RNA was converted to cDNA by treatment with 200 units of reverse transcriptase and 500 ng of oligo(dT) primer in 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, and 1 mM dNTPs at 42 °C for 1 hour. The reaction was stopped by heating at 70 °C for 15 min. One μl of the cDNA mixture was used for enzymatic amplification. The polymerase chain reaction was performed in 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 2.5 units of *Taq* DNA polymerase, and 0.1 μM of primers for MMPs and TIMPs. Amplification was performed in a DNA thermal cycler (Model PTC-200, MJ Research, Burlington, USA) under the following conditions: denaturation at 94 °C for 5 min for the first cycle and for 30 seconds thereafter, annealing at 55 °C (MMPs and TIMPs) for 30 seconds, and extension at 72 °C for 30 seconds for total of 25 repetitive cycles. Final extension was at 72 °C for 10 min.

4. Western blot analysis

To detect MMPs and TIMPs, western blot was performed. The proteins were separated according to their molecular weights by the SDS–PAGE (sodium dodecyl sulphate–polyacrylamide gel electrophoresis). Immunoblotting was conducted using polyvinylidene difluoride membranes (Millipore, Bedford, MA) and the transfer was carried out for 1 hour at 225 mA/ membrane, at 100 V. The membranes were blocked with 5% skim milk in 20mM Tris, 500mM NaCl and 1% Tween-20 (pH 7.5) for 2 hours. And then the membranes were subsequently incubated for 2 hours at room temperature with monoclonal primary antibodies directed at MMP2, MMP9, TIMP-1 and TIMP-2 (1:100, Calbiochem, San Diego, CA), respectively. After washing, the membranes were incubated with secondary antibodies (anti-rabbit polyclonal IgG, Horseradish peroxidase conjugated anti- goat polyclonal IgG: Santa Cruz Biotechnology, CA, USA; Anti-mouse monoclonal IgG : Amersham, Buckinghamshire, England) anticoupled to alkaline phosphatase followed by detection with enhanced chemiluminescence (ECL, Amersham, Buckinghamshire, England). The blots were visualized with densitometric scanning using the densitometer (IMAGE READER LAS-1000 lite, Fuji Photo Film Co., Ltd., Japan) with the digital analysis software (Fuji Photo Film Co., Ltd., Japan).

5. Gelatin zymography

Preeclamptic villous tissues after being cultured at 3% oxygen for 5 days were washed twice with serum-free medium and grown in serum-free medium for 6 hours before the addition of any agent. First, to analyze dose-dependent

secretions of MMPs by adenosine A3 agonists, cultures were stimulated with Cl-IB-MECA at 0, 10 and 100nM, respectively, for 12 hours. Second, cultures were preincubated for 1 hour with or without adenosine A3 antagonists MRE3008F20 (100nM). Thereafter, to analyze the time course expressions of MMPs, cultures were treated with Cl-IB-MECA (100nM) for 36 hours.

The supernatant was collected to assess gelatinase activity. The samples were electrophoretically separated onto 8.5% SDS–polyacrylamide gel containing 1 mg/ml of gelatin (Sigma, St. Louis, MO, USA). After electrophoresis, the gel was washed at room temperature for 1 hour in washing buffer (50 mM Tris-Cl, pH 7.4 and 2.5% Triton X-100) and incubated at 37 ^{0}C overnight in incubation buffer (50 mM Tris–Cl, pH 7.4, 75 mM NaCl and 2.5 mM CaCl2). The gel was stained with 0.2% Coomassie brilliant blue R-250 (Sigma St. Louis, MO, USA) in a mixture of methanol: acetic acid: water (2: 1: 7) for 2 hours and then destained in the same solution without the dye. Clear zones against the blue background indicated the presence of gelatinolytic activity.

6. Statistics

All values are presented as mean±standard error. Statistical comparisons were performed using a Student *t*-test and analysis of variance (ANOVA). Data analysis was performed using the Statistical Package for Social Sciences for Windows, version 12.0 (SPSS Inc., Chicago, Ill, USA). A p value less than 0.05 was considered statistically significant.

III. RESULTS

1. Expression of A3R, MMP-2/-9, and TIMP-1/-2 in villous explants before culture

Preliminary experiments of sqRT-PCR and Western blot were performed to determine the expression of A3R, MMP-2/-9, and TIMP-1/-2 in normal and preeclamptic villous explants before being cultured. The signal intensity of A3R was significantly increased whereas the signal intensities of MMP-2/-9 and TIMP-1/-2 were reduced in preeclamptic villous explants compared to those of normal villous explants (Fig. 1).

2. Expression of A3R, MMP-2/-9, and TIMP-1/-2 in normal villous explants after culture at high and low oxygen

Thereafter, both explants were cultured at high and low oxygen level for 5 days. In normal villous explants, after being cultured at high oxygen level for 5 days, the signal intensity of A3R, MMP-2/-9, and TIMP-1/-2 were similar to those before culture. However, the signal intensity of A3R was significantly increased whereas the intensities of MMP-2/-9 and TIMP-1/-2 were reduced at low oxygen level compared to those at high oxygen level (Fig. 2A). Such findings are comparable to those of preeclamptic villous explants in our earlier studies.

3. Expression of A3R, MMP-2/-9, and TIMP-1/-2 in preeclamptic villous explants after culture at high and low oxygen

In preeclamptic villous explants, after being cultured at high oxygen level for 5 days, we found a relatively weak signal intensity of A3R, and strong intensity of both MMP-2/-9 and TIMP-1/-2. Similar signal patterns were seen in those of normal villous explants from our preliminary experiments. Interestingly,

significantly increased signal intensity of A3R and reduced intensity of MMP-2/-9 and TIMP-1/-2 were shown in culture at low oxygen level compared to those cultured at high oxygen level (Fig. 2B).

4. Time- and dose-dependent secretion of MMP-2/-9 by adenosine A3 receptor in preeclamptic villous explants at low oxygen level

A3R agonist, Cl-IB-MECA, was treated in a dose-dependent manner in preeclamptic villous explants after being cultured at low oxygen level for 5 days. Treatment of preeclamptic villous explants with Cl-IB-MECA resulted in an enhanced secretion of MMP-2 and MMP-9 at control, 10nM, and 100nM in a dose-dependent manner (Fig. 3).

Also, time-dependent secretions of MMP-2 and MMP-9 were shown with 100nM of Cl-IB-MECA (Fig. 4). In the presence of Cl-IB-MECA (100nM), MMP-2 levels were significantly increased at 4 hours, reaching maximal level at 12 hours, continued to stay stable up to 24 hours and thereafter, gradually returned to control levels. Similar secretion pattern of MMP-9 appeared but gradually decreased after reaching maximal level at 12 hours. Pretreatment of A3R antagonist, MRE3008F20 (100nM), for 1 hour significantly inhibited Cl-IB-MECA (100nM)-induced secretion of MMP-2 and MMP-9 (Fig. 4).

Table 1. Primer sequences specific to the target genes

Gene	Dir	Sequence	Size(bp)
MMP-2	s	5’-AGGACATTGTATTTGATGGC-3’	326
	a	5’-CTTCTTGTTTTTGCTCCAGT-3’	
MMP-9	s	5’-AGCTTTTCTTCTTCTCTGGG-3’	378
	a	5’-ACTGCAGGATGTCATAGGTC-3’	
TIMP-1	s	5’-CACCAGAGAACCCACCATG-3’	583
	a	5’-GCAGGCTTCAGCTTCCACTC-3’	
TIMP-2	s	5’-TTTGCAATGCAGATGTAGTG-3’	535
	a	5’-TCGAGAAACTCCTGCTTGG-3’	
GAPDH	s	5'- CGCCACAGTTTCCCGGAGGG -3'	346
	a	5'- CCCTCCAAAATCAAGTGGGG -3'	

Dir, direction; S, sense; a, antisense; bp, base pair.

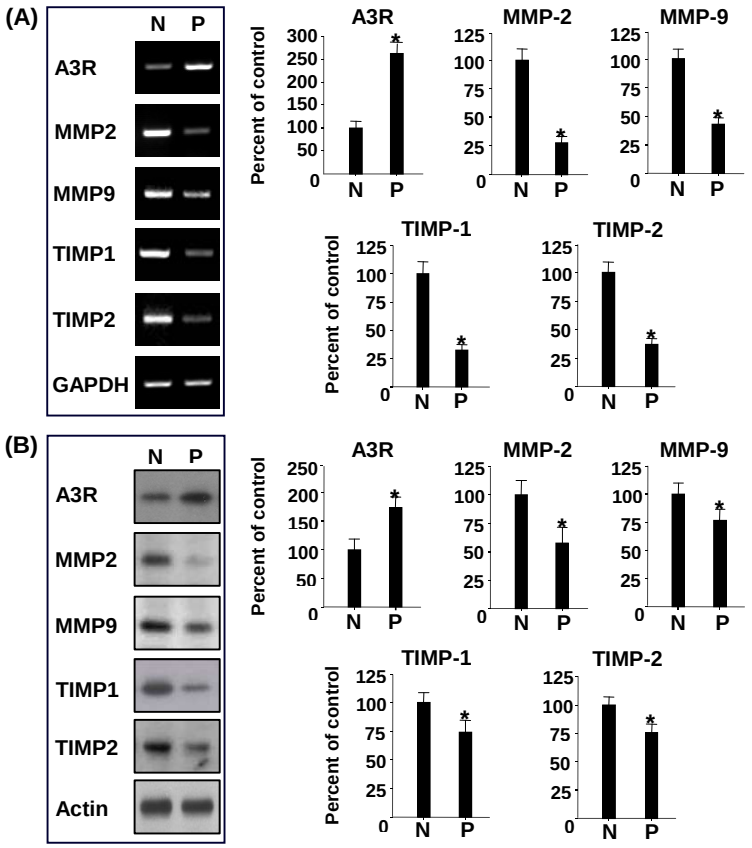

Figure 1. Adenosine A3 receptor (A3R), MMP-2/-9, and TIMP-1/-2 expressions in normal (N, n=10) and preeclamptic (P, n=10) villous explants before culture. Total mRNA and protein were isolated from preeclamptic and normal villous explants. And then genes expression was measured by sqRT-PCR (A) or Western blot analysis (B). sqRT-PCR and Western blot analysis revealed that the intensity of A3R was significantly increased whereas the intensities of MMP-2/-9 and TIMP-1/-2 were reduced in preeclamptic villous explants compared to those of normal villous explants. Data are means ± SD of densitometry measurements relative to the results obtained in normal villous explants (control set at 100%). *p<0.05 compared with the control.

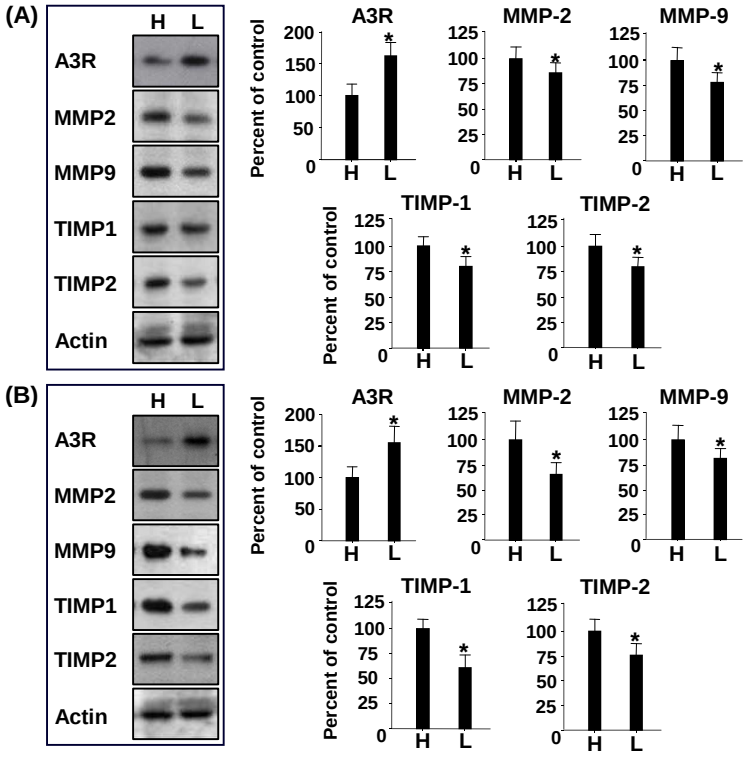

Figure 2. Western blot analysis of adenosine A3 receptor (A3R), MMP-2/-9, and TIMP-1/-2 expressions after being cultured at 20% oxygen (H) and 3% oxygen (L) for 5 days. Preeclamptic and normal villous explants were cultured at 20% oxygen and 3% oxygen. After 5 days, total protein was isolated and Western blots were performed. The signal intensity of A3R was significantly increased whereas the intensities of MMP-2/-9 and TIMP-1/-2 were reduced at low oxygen level compared to those at high oxygen level in normal villous explants (n=10) (A). Significantly increased signal intensity of A3R and reduced intensity of MMP-2/-9 and TIMP-1/-2 were shown at low oxygen level compared to those cultured at high oxygen level in preeclamptic villous explants (n=10) (B). Data are means ± SD of densitometry measurements relative to the results obtained after being cultured at 20% oxygen (control set at 100%). *p<0.05 compared with the control.

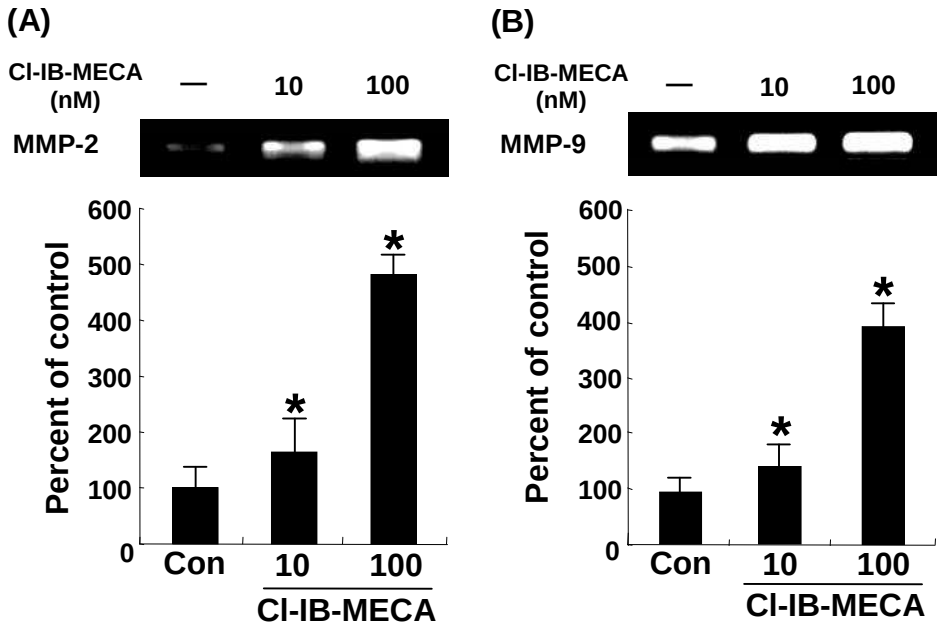

Figure 3. Induction of MMP-2/-9 by A3R agonist, CI-IB-MECA (control, 10nM, and 100nM). MMP-2 (A) and MMP-9 (B) by zymogram assay in preeclamptic villous explants (n=10) after being cultured at 3% oxygen for 5 days. Treatment of preeclamptic villous explants with A3R agonist for 12 hours resulted in an enhanced secretion of MMP-2 and MMP-9 in a dose-dependent manner. Data are means ± SD of densitometry measurements relative to the results obtained in hypoxic culture in the absence of CI-IB-MECA (control set at 100%). *p<0.05 compared with the control.

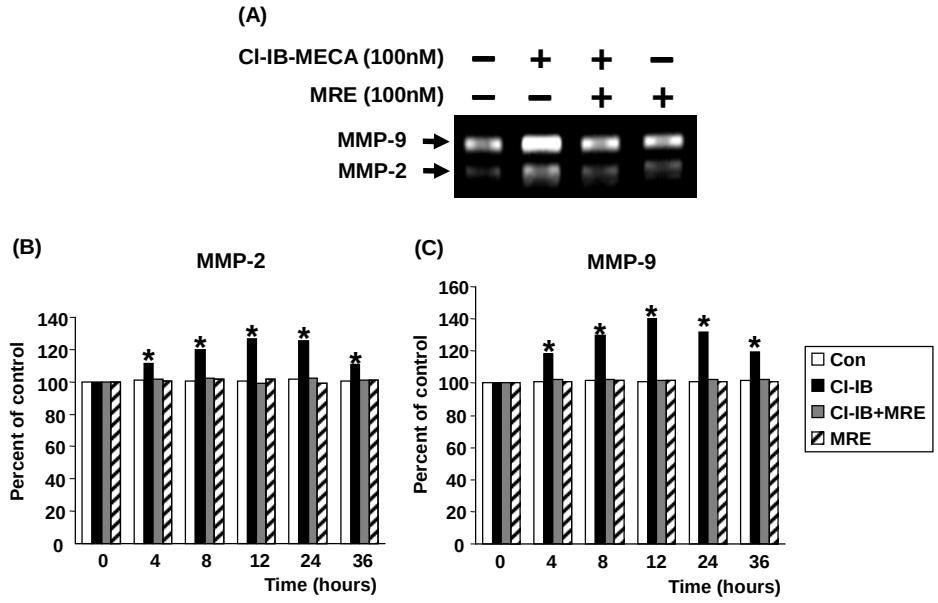

Figure 4. Time course expression of MMP-2/-9 by A3R agonist, CI-IB-MECA (100nM), pretreated with (+) or without (-) A3R antagonist, MRE3008F20 (100nM). Representative MMP-2 and MMP-9 zymogram assay of concentrated media from preeclamptic villous explants (n=10) at 12 hours after being cultured at 3% oxygen for 5 days (A). Black bars show that MMP-2/-9 levels reached maximal level at 12 hours in the presence of Cl-IB-MECA (100nM). Gray bars show that pretreatment of A3R antagonist, MRE3008F20 (100nM), for 1 hour significantly inhibited Cl-IB-MECA (100nM)-induced secretion of MMP-2/-9 (B, C). Data are means of densitometry measurements relative to the results obtained at time 0 of experiments (control set at 100%). *p<0.05 compared with the control. Con, control; CI-IB, CI-IB-MECA; MRE, MRE3008F20.

IV. DISCUSSION

The objective of this study was to evaluate the modulation of MMP secretion by adenosine A3 receptors in normal and preeclamptic villous explants. The major findings of this study are as follows: 1) The signal intensity of A3R was significantly increased and the intensities of MMP-2/-9 and TIMP-1/-2 were reduced in preeclamptic villous explants compared to those of normal villous explants before culture; 2) in normal villous explants, significantly increased A3R signal intensity and reduced MMP-2/-9 and TIMP-1/-2 intensities at low oxygen level were noted compared to those at high oxygen level; 3) in preeclamptic villous explants, i) after being cultured at high oxygen level for 5 days, the signal intensity of A3R were relatively weak, and the signal intensities of MMP-2/-9 and TIMP-1/-2 were strong as seen in normal villous explants in our preliminary experiments, ii) significantly increased intensity of A3R and reduced intensity of MMP-2/-9 and TIMP-1/-2 were shown in cultures at low compared to high oxygen level; 4) treatment of preeclamptic villous explants with Cl-IB-MECA resulted in dose and time-dependent secretion of MMP-2 and MMP-9.

The interaction of invading trophoblast with the uterine vasculature is an essential feature of human placentation. Interaction of trophoblast with the extracellular matrix is one of the main factors in providing a substrate for attachment, growth and/or migration. Inadequate trophoblast invasion and deficient remodeling of uterine spiral arteries are associated with preeclampsia.[15] These deficiencies of placentation are postulated to cause focal regions of hypoxia that, in turn, stimulate the overproduction of various placental products, such as proinflammatory cytokines, that spill over into the maternal circulation, thereby causing endothelial dysfunction and systemic disease.[34]

Previous studies have shown that human trophoblast invasiveness in vitro depends on the production of MMPs and that both MMP-2 and MMP-9 are secreted by human trophoblasts isolated from first trimester placenta.[25] It has also shown that trophoblast from third trimester placenta secretes primarily MMP-9 and minimal amounts of MMP-2.[28] Consistent with our findings, the signal intensity of MMP-9 was stronger than that of MMP-2 in normal villous explants before culture. Furthermore, reduced signal intensity of MMP-2/-9 in preeclamptic villous explants before culture can partially explain the maladaptation of uterine spiral arteries through impaired implantation and inadequate trophoblast invasion in preeclampsia.

TIMPs are the major endogenous inhibitors of MMP activities in tissues.[18] Binding of TIMPs to the catalytic domain results in efficient inhibition of the enzymatic activity of MMPs. TIMPs generally inhibit the activity of MMPs by the formation of a 1:1 complex. Our study demonstrated that MMPs and TIMPs were coexpressed in villous explants. The signal intensities of TIMPs in our study have similar patterns with those of MMPs, suggesting that the expression of TIMPs could be increased as a compensatory mechanism to the increased expression of MMPs.

Adenosine is an ubiquitous autacoid that accumulates to high levels in hypoxic tissues as a result of ATP breakdown.[2] Therefore, this nucleoside could be involved in the regulation of the cellular response to hypoxia. In particular, it is recognized that significant levels of adenosine are present in the extracellular fluid of solid tumors,[35] suggesting a role for this autacoid in tumor growth. Interestingly, recent data indicate that A3 receptor overexpression may be a good candidate for a tumor cell marker.[36] Primary and metastatic tumor tissues express A3AR indicating that high receptor expression is a characteristic of solid tumors. These findings suggest A3AR as a potential target for tumor growth inhibition.[37]

Another important evidence of cellular hypoxia is increased hypoxia-inducible factor-1 (HIF-1). HIF-1 activates the transcription of genes that are involved in crucial aspects of cancer biology, including angiogenesis, cell survival, glucose metabolism, and invasion.[38] Intratumoral hypoxia and genetic alterations can lead to HIF-1α overexpression, which in turn, has been associated with increased patient mortality in several types of cancer. In preclinical studies, inhibition of HIF-1 activity has produced a remarkable effect on tumor growth. Efforts are on the way to identify inhibitors of HIF-1 and to test their efficacy as anticancer therapeutics. Moreover, protein expression of HIF-2a, but not of HIF-1α or -1β, was selectively increased in the preeclamptic placenta compared to normal term placenta.[39] An obvious explanation for the selective increase of HIF-2a in preeclamptic placenta is that the elevated levels of HIF-2a reflect hypoxia which is believed to be an important pathogenesis in many preeclamptic placentas, also seen in several aspects of cancer biology.

Recently, we have found significantly increased expressions of adenosine A3 receptors in trophoblasts of pregnancies complicated by preeclampsia.[32] Immunohistochemical staining revealed that adenosine A3 receptors were not present in enothelial cells, but were seen only in trophoblasts, and the staining intensities were stronger in preeclampsia than in the normal group. Together with the increased HIF seen in previous studies, our study supports the notion that hypoxia is an important mechanism in the placenta of pregnancies destined to become preeclamptic. Therefore, the purpose of this study was to investigate the relationship between MMP secretion and the specific types of elevated adenosine receptor subtypes under hypoxic conditions in vitro and ultimately to investigate the possible role of adenosine receptor as a therapeutic target in preeclampsia.

In this study, the expression of A3R from normal villous explants was increased and that of MMP-2/-9 decreased in low oxygen condition as seen in

our preliminary study on preeclamptic villous explants. However, when the oxygen concentration is altered from low to high, the expression of A3R from preeclamptic villous explants was decreased and that of MMP-2/-9 was increased as seen in our initial study on normal villous explants. From these results, we can conclude that protein expression of villous explants may be reversible in relation to oxygen concentration. Therefore, if the hypoxic environment of preeclamptic placenta is altered to a rich oxygen environment, or if substances could be provided to increase oxygen concentrations as a compensatory mechanism to augment MMP secretion, it would be an astonishing achievement for opening a new chapter in treatment of preeclampsia.

Adenosine and its specific receptor subtype A3R which increase in trophoblasts under hypoxic environment were studied to determine its ability to control MMP secretion. When preeclamptic villous explants were treated with A3R agonist, Cl-IB-MECA, after 5 days of culture under hypoxic condition, dose and time-dependent secretion of MMP-2 and MMP-9 were shown and pretreatment of A3R antagonist, MRE3008F20 significantly inhibited Cl-IB-MECA-induced secretion of MMP-2 and MMP-9. These results demonstrate that the effects are A3R specific.

The molecular mechanism of MMP secretion modulated by adenosine has not yet been identified. Adenosine, known to be an important modulator of immune response through inflammatory or anti-inflammatory effect, especially plays an essential role in inhibiting the cytokine production. The inflammatory cytokines, tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β), are notorious for producing endothelial dysfunction, and interestingly, synthesis of these cytokines, as well as IL-6, by trophoblast and other cells of the normal human placenta has been documented.[40-42] The term placenta produces TNF-α,

IL-6, and low levels of IL-1α and IL-1β under standard tissue culture conditions. Hypoxia significantly increased TNF-α, IL-1α, and IL-1β production by 2-, 6-, and 23-fold, respectively, but did not affect IL-6 production in villous explants from the human term placenta.[43] Furthermore, cytokines were immunolocalized to the syncytiotrophoblast layer as well as to some villous core cells. Vascular endothelial growth factor (VEGF) as well as inflammatory cytokines, have been implicated in the pathophysiology of preeclampsia and all are capable of stimulating the release of MMPs.[44-46] Adenosine also plays a role in the promotion of angiogenesis.[47] Regulation of expression of the angiogenic VEGF via adenosine receptors has been demonstrated in several cell types.[48,49] Recent studies have demonstrated molecular mechanisms in which A3 receptor stimulation induces increase of HIF-1 and VEGF by activating p44/p42 and p38 mitogen-activated protein kinases (MAPK).[50] Based on the current review of the literature, adenosine production could be increased in a dose-dependent manner under hypoxic environment, as a compensatory mechanism to the increase of cytokines. The increased cytokines, VEGF, etc. then eventually enhance the secretion of MMP.

This study used the term villous explants that are relatively easy to obtain as study samples. Additional studies are needed in order to determine the possibility of MMP modulation by specific A3 receptor in the first trimester villous explants and to see if the A3 agonists could modify the invasiveness of trophoblasts in vivo.

V. CONCLUSION

The major findings of this study are as follows:

1) The signal intensity of A3R was significantly increased and the intensities of MMP-2/-9 and TIMP-1/-2 were reduced in preeclamptic villous explants compared to those of normal villous explants before culture;

2) In normal villous explants, significantly increased A3R signal intensity and reduced MMP-2/-9 and TIMP-1/-2 intensities at low oxygen level were noted compared to those at high oxygen level;

3) In preeclamptic villous explants i) after being cultured at high oxygen level for 5 days, the signal intensity of A3R were relatively weak, and the signal intensities of MMP-2/-9 and TIMP-1/-2 were strong as seen in normal villous explants in our preliminary experiments ii) significantly increased intensity of A3R and reduced intensity of MMP-2/-9 and TIMP-1/-2 were shown in cultures at low oxygen level compared to those at high oxygen level;

4) Treatment of preeclamptic villous explants with Cl-IB-MECA resulted in dose and time-dependent secretion of MMP-2 and MMP-9.

To the best of our knowledge, this is the first study to evaluate the modulation of MMP secretion by adenosine A3 receptor in preeclamptic villous explants at term. Our results provide evidence for the existence of functional adenosine A3 receptor in the trophoblast and suggest that adenosine A3 receptor could be used as a therapeutic target in preeclampsia with further investigation.

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<ABSTRACT (IN KOREAN)>

전자간증 태반 융모배양물에서 아데노신 A3 수용체에 의한 Matrix Metalloproteinase 분비 조절

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연구목적: 조직 허혈이나 염증 부위에서 분비되는 아데노신(adenosine)은 중요한 생물학적 역할을 가진다. 현재까지 A1, A2a, A2b, A3 의 4가지 아데노신 수용체가 있는 것으로 알려져 있다. 최근 전자간증 임신의 영양막 세포에서 아데노신 A3 수용체가 증가되어 있음이 보고되었으며 이러한 사실은 비혈관적으로 영양막 세포를 경유하여 아데노신 A3 수용체가 전자간증의 병인에 중요한 역할을 할 수 있음을 시사한다. 전자간증 임신에서 matrix metalloproteinase(MMP) 와 연관된 영양막 세포의 부적절한 침습과 여러 다른 영역에서 아데노신 수용체와 MMP의 관련성이 보고되었다. 본 연구는 다른 산소 농도 배양 조건 하 전자간증 태반 융모배양물에서 아데노신 A3 수용체에 의한 MMP 분비 조절에 대해 알아보고자 하였다.

연구방법: 만삭의 정상 임신 및 전자간증 임신의 태반 융모배양물을 각 10례를 얻어 5일 동안 고농도 및 저농도 산소 조건하에 배양하였다. 융모배양물에서 MMP-2/-9 와 TIMP-1/-2 는 RT-PCR 과 Western blot 방법을 이용하여 분석하였다. 저농도 산소 조건하에서 배양된 전자간증 태반 융모배양물에 A3 수용체의 작용제인 Cl-IB-MECA 와 길항제인 MRE 를 처치하였다. 이후 RT-PCR, Western blot, 그리고 zymography 방법을 이용하여 MMP-2/-9 의 작용제 농도 및 노출 시간에 따른 분비 양상을 분석하였다.

결과: 저농도 산소 조건에서 배양된 정상 및 전자간증 태반 융모배양물에서 유의한 A3 수용체 증가 및 MMP-2/-9, TIMP-1/-2 감소를 보였다. 흥미롭게도 고농도 산소 조건에서 배양된 전자간증 태반 융모배양물에서 MMP-2/-9 와 TIMP-1/-2 는 배양 전 정상 태반 융모배양물에서 나타나는 결과와 유사한 양상을 보여 이러한 사실은 가역적인 변화가 나타날 수 있슴을 시사한다. 저농도 산소 조건하에서 배양된 전자간증 태반 융모배양물에 A3 수용체의 작용제인 Cl-IB-MECA 를 처치하였더니 작용제의 농도 증가 및 노출 시간에 따라 MMP-2/-9의 분비의 유의한 증가가 나타났다. 이러한 양상은 길항제인 MRE 를 전처치함으로써 모두 억제되었다.

결론 : 본 연구는 전자간증 태반 융모 배양물에서 아데노신 A3 수용체에 의한 MMP 분비 조절에 관한 최초의 연구로 영양막 세포에서 기능적인 아데노신 A3 수용체가 존재하며 향후 아데노신 A3 수용체가 전자간증 임신의 치료제로 연구될 수 있는 증거를 제시하였다.

핵심되는 말 : 아데노신 A3 수용체, matrix metalloproteinase, 전자간증, 태반 융모배양물