Neuroprotective effects of astrocytes expressing ADC gene on oxygen-glucose deprivation injury

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Neuroprotective effects of astrocytes expressing ADC gene on oxygen-glucose deprivation injury

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멋진 여성상의 본보기이시며 우아한 카리스마를 지니신 박경아 교수님. 교수님의 첫 수업을 듣던 때를 기억합니다. 눈부신 교수님의 포스는 언제나 저의 동경의 대상이 되었던 것 같아요. 주신 가르침들 감사드립니다.

해부학교실의 아버지이자 저에게 언제나 인자하게 대해주신 이원택 교수님과 바쁘신 일정 속에서도 저의 논문을 지도해 주신 신경외과 허지회 교수님께 감사의 말씀을 드립니다.

많은 시간을 함께 보내며 도움주신 신영호 선생님 이하 연구실 식구들에게도 고마움을 전합니다. 특히, 실험 할 수 있는 손을 만들어주시고 많은 조언을 주셨던 김종열 선생님과 언제나 꼼꼼하게 실험적 노하우를 가르쳐주신 최윤정 선생님. 이 두 분께 감사의 말씀을 전하고 싶습니다. 그리고 한참 과학적 지식이 절실할 타이밍에 큰 도움을 주신 김재환 박사님께도 너무나 감사드립니다. 늘 수고가 많은 재영이, 언제나 친절한 연옥언니, 그리고 착한 마음씨를 가진 후배 윤미에게 고마움을 전합니다. 언제나 안부를 챙기며 조언해주시고 순간순간을 가이드해주신 안수경 선생님께도 진심으로 감사드립니다. 이따금씩 냉철한 한마디씩을 남겨주는, 언제나 그 누구보다 우월했던 나의 youngest mento 호원이. 고맙다. 또한 꽃다운 나이에 연구실 창밖너머의 낭만적인 여러 계절을 뒤로한 서로의 모습을 위로하며 수고를 나눈 약리학 임영신 선생님에게도 고맙습니다.

같은 시기에 학위를 하고 있음에도 언제나 나의 편에 서주시고 또한 나의 앞 과 뒤를 냉정하게 판단해 주며 진심으로 힘이 되어주신 상원오빠에게 감사드립니다. 그리고 언제나 서로를 자랑스럽게 여기고 어느 상황에서건 서로의 편에서 생각해주는 든든한 나의 두 벗. 가족 같은 친구들인 지혜와 양미에게도 고마운 마음을 전합니다.

이 세상에서 나와 가장 동일한 DNA를 가진 하나뿐인 남동생 손대근 에게 깊은 사랑과 고마운 마음을 담습니다. 동생에게 힘을 줄 수 있는 누나가 되어야 한다는 막연한 사명감은 언제나 제게 큰 동기부여가 되어 주었던 것 같습니다.

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끝은 곧 시작이라 배웠습니다.

앞으로 더더욱 노력해야할 나의 수고들에 격려의 박수를 보냅니다.

2009 년 1월

손 미 란

네가 큰 일을 행하겠고 반드시 승리를 얻으리라.

You will do great things and surely triumph.

(사무엘상 26:25)

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ABSTRACT

Neuroprotective effects of astrocytes expressing ADC gene on oxygen-glucose deprivation injury

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Agmatine is an endogenous amine derived from the decarboxylation of arginine catalysed by arginine decarboxylase(ADC), and metabolized to putrescine by agmatinase. Agmatine is considered a novel neuromodulator and possesses neuroprotective properties in the central nervous system. In the

previous study, it has reported that agmatine reduced infarct volume and attenuated brain edema from cerebral ischemic injury when they administered exogenously. In this study, it has been investigated that the effect of agmatine administered endogenously through overexpression of ADC genes which are a synthesizing enzyme of agmatine using retroviral vector on brain injury. For this purpose, a retroviral vector containing ADC genes was transfected to primary cultured astrocytes. The expression of ADC in primary cultured astrocytes was detected by immuocytochemistry using ADC antibody. The level of agmatine generated endogenously was measured by HPLC. It has then demonstrated whether endogenous agmatine protect cells from OGD injury in astrocytes overexpressed ADC genes.

Matrix metalloproteinases(MMPs) are up-regulated by ischemia and degrade the basement membrane of brain vessels to promote cell death and tissue injury. And, it has been reported that neuroprotective effects of agmatine is associated with decreased NOS expression. The iNOS expressed in the endothelial cells and nitric oxide generated by this enzyme play an important role in regulation of vascular reactivity. So, their inhibition is neuroprotective and may be a useful therapeutic strategy to target selectively the progression of ischemic brain injury.

In this study, it has investigated whether endogenous agmatine, produced by ADC genes, decrease the MMPs expression in astrocyte. RT-PCR, westernblot and immunocytochemical analysis against MMP-2, MMP-9, iNOS and caspase-3 were performed for this study. It has shown that endogenous agmatine, produced by ADC genes, decreased the number of iNOS, caspase3, MMP2 and MMP9 positive cells after oxygen-glucose deprivation injury. It may suggest that agmatine administered endogenously using viral vector system shows neuroprotective effects through reducing the expression of iNOS, caspase-3, MMP-2 and MMP-9 in astrocytes under ischemic-like injury.

This decreases may explain the protective mechanism of endogenous agmatine after ischemia.

Key words : arginine decarboxylase(ADC), agmatine, astrocyte, ischemia, neuroprotection

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I. INTRODUCTION

Agmatine, a naturally occurring guanidino compound found abundance in bacteria and plants, and was recently identified in mammalian brains also. Agmatine is an endogenous polyamine derived from enzymatic decarboxylation of L-arginine by arginine decarboxylase (ADC)[3,4]. It is hydorolyzed to

putrescine and urea by agmatinase. Agmatine is an endogenous clonidine-displacing substance, an agonist for the α2-adrenergic and imidazoline receptors, and an antagonist for the N-methyl-D-aspartate (NMDA) receptors[7]. ADC is widely expressed in many brain areas of rats and humans. Recent studies have shown that agmatine may be neuroprotective in neurotrauma, neonatal ischemia models and cultured neurons[15,18]. Agmatine is synthesized, stored in astrocytes, and released from specific networks of neurons.

Astrocytes play a crucial role in the central nervous system (CNS)[8,24]. They modulate synaptic transmission and plasticity, guide axonal growth during development and secrete growth factors to support neuronal survival[8,24]. Therefore, dysfunction or loss of astrocytes can lead to neuronal death or dysfunction. Since astrocytes play a central role in maintaining neuronal viability both under normal conditions and during stress such as ischemia, its function to stress is essential to understand various brain pathology. A significant amount of brain damage caused from ischemic stroke can be attributed to neuronal cell death, resulting from an insufficient supply of glucose and oxygen to brain tissue. To understand the mechanisms of neuronal cell death after ischemic insult and to identify potential protective agents, *in vitro* cell culture model of ischemia has been developed[15,18,50].

Nitric oxide (NO) is known to trigger and mediate cascades involved in inflammation and apoptosis in ischemic injury and iNOS is also involved in the mechanisms by which ischemia-induced apoptosis. iNOS is expressed predominantly in inflammatory cells infiltrating the ischemic brain[43,47]. Excessive amount of NO derived from reactive astrocyte is assumed to contribute neuronal death during ischemia, trauma, and oligodendrocyte degeneration in demyelinating diseases. So, their inhibition is neuroprotective and may be a useful therapeutic strategy to target selectively the progression of ischemic brain injury[25,42,43].

The extracellular matrix molecules constitute the basement membrane underlying the vasculature and play a critical role for providing structural support to cell wall. Matrix metalloproteinases(MMPs) are a group of zinc-dependent enzymes that may contribute to the pathophysiology of cerebral ischemia by degrading the matrix components in the neurovascular unit. An early and major pathological effect of MMPs in cerebral ischemia is the disruption of the blood-brain barrier (BBB) through the degradation of basal lamina that surround the cerebral blood vessels[51,52,55]. Members of the MMP family, MMP-2 and MMP-9 are well-known, which degrade the extracellular matrix components of the basement membrane of brain vessels[56,57].

The present study was aimed to evaluate whether endogenous agmatine, produced by ADC genes, modulate the neuroprotection *in vitro* using astrocyte from ischemic injury. This is achieved by investigating the level of agmatine generated endogenously by HPLC, and also aimed to determine whether endogenously administered agmatine decrease caspase-3, iNOS, MMP-2 and MMP-9 expression after oxygen-glucose deprivation(OGD) injury in astrocytes. Accordingly, it may suggest that endogenous agmatine in the astrocyte expressing ADC gene may play a permissive role through reduced the degree of cell death in neuronal pathology induced by OGD stress.

II. MATERIALS AND METHODS

1. Primary astrocyte cultures

Primary cortical astrocytes were cultured from 1 to 3 day old postnatal ICR mouse, and maintained in minimum essential medium (MEM, Gibco) containing 10% fetal bovine serum and 10% equine serum (Hyclone). Briefly, hemispheres of newborn ICR mouse were removed aseptically from the skulls, freed of the menings. The cortex was dissected, treated with 0.09% trypsin for 20 min at 37℃, and triturated. Cells were plated onto 6-well and 24-well plates at a density of 1 hemisphere per plate and the culture medium was changed twice a week for a period of 20 days.

2. ADC infection

For this study, we constructed the retroviral vector containing human ADC cDNA and established a transfection of human ADC gene into the astrocyte(AST-ADC) by the retroviral packaging cell line PT67.

3. Oxygen-glucose deprivation injury

Cultures were transferred into an anaerobic chamber (Forma Scientific Co.) (O2 tension 〈0.2%), washed three times in deoxygenated, glucose-free balanced salt solution (BSS_0) containing 116mM NaCl, 1.8mM $CaCl_2$, 0.8mM $MgSO_4$, 5.4mM KCl, 1mM NaH_2PO_4, 14.7mM $NaHCO_3$, 10mM HEPES and 10mg/L phenol red at PH 7.4 and incubated in BSS_0 at an oxygen-free incubator for 4hrs. Following OGD, glucose is added to the culture medium to a final concentration of 5.5mM. And, cells were incubated under normal growth conditions for indicated time.

4. Measurement of lactate dehydrogenase activity

Cell lysis was quantified by lactate dehydrogenase assay (LDH) released into the culture medium. The amount of total LDH released of 100% cell death named "full kill" was determined at the end of experiment following freezing at -70℃ and rapid thawing. The extent of cell death was expressed as percentage of full kill.

5. Hoechst-PI nuclear staining

Cell death was evaluated morphologically by staining the non-viable cell with propidium iodide(Sigma, St. Louis, Missouri, USA), and living cells with Hoechst 33258 dye(Sigma, St, Louis, Missouri, USA). Staining with the fluorescent dyes propidium iodide and Hoechst 33258 allows discrimination of apoptotic from non-apoptotic cells on the basis of nuclear morphology and evaluation of membrane integrity. Hoechst dye was added to the culture medium(2-5 ㎍/ml) and then cells were kept at 37℃ for 30 minutes. Propidium iodide solution was then added(2-5 ㎍/ml) just before observation in a Olympus microscope equipped with epifluorescence and a UV filter block.

6. HPLC (High Performance Liquid Chromatography)

The amount of agmatine in control and transfected cells was measured by HPLC. The harvested cells were homogenized in phosphate buffer containing internal standard and 10% trichloroacetic acid (TCA), centrifuged at 12000 rpm for 10 min. The supernatant was mixed with *o*-pthalaldehyde (OPA) and injected into the HPLC system with column and detected by fluorescence detector. Recovery of agmatine was calculated from the added external standard and expressed as ng/mg protein.

7. Immunocytochemistry

Cells on culture plated which were coated with poly-D-lysine/laminin and were washed three times with PBS, fixed with 90% Ethanol for 30 minutes on ice, and then washed three times with TBS. Cells were permeabilized with 1.6% H_2O_2 in TBS and 0.025% triton, and cells were blocked for 1 hour at room temperature with 0.5% bovine serum albumin and 6% normal goat serum in TBS. Primary antibodies rabbit anti-iNOS (1:500), rabbit anti-caspase3 (1:250), mouse anti-MMP2(1:500), mouse anti MMP9(1:500), mouse anti-GFAP(1:500) were then added in TBS containing 3% normal goat serum and left overnight at 4℃. Primary antibody was removed, by washing the cells three times for 5 minutes each with TBS. Appropriate secondary antibody was conjugated to CY3 or fluorescein isothiocyanate was added in TBS containing 1% BSA for 2 hours at room temperature (FITC, Zymed, 1:1000/ Rhodamine, Zymed, 1:500). Cells were washed three times for 5 minutes each with TBS, conterstained with 4'-6-Diamidino-2-phenylindole (1㎍/ml, DAPI, Sigma) for 30 minutes at room temperature, and cells were visualized using a Olympus D-70 upright fluorescent microscope.

8. Protein extraction

Primary cultured astrocytes were washed with ice-cold PBS and harvested cells were solubilized with buffer A [10mM HEPES, pH 7.4, 10mM KCl, 0.1mM EDTA, 0.1mM EGTA, 1mM DTT, 1μg/mL leupeptin, 1μg/mL aprotinin, 1mM PMSF]. After 15min on ice, Igepal were added to the lysates to a final concentration 0.5%. The tubes were vigorously vortexed for 10sec and centrifuged at 14000 rpm for 30sec. The supernatants were stored at -80℃ (cytosolic fraction), and the nuclei pellet was resuspended with buffer B [20mM HEPES, ph 7.4, 400mM NaCl, 1mM EDTA, 10% glycerol, 1mM DTT,

1μg/mL leupeptin, 1μg/mL aprotinin, 1mM PMSF]. The nuclei solution incubated on ice for 30min vortexing for every 10min, nuclear proteins were obtained by centrifugation at 14000rpm for 15min, and the supernatants were stored at -80℃. Protein concentration was quantified using the BCA protein assay kit (Pierce, Rockford, IL, USA).

9. Western blot analysis

Equal amounts of protein were subjected to electrophoresis on 10% SDS-polyacrylamide gels. Separated proteins were then electro-transferred to Immobilion-NC membrane (Millipore, Bedford, MA, USA). The membranes were blocked for 1h at room temperature with 5% skimmed milk in TBS and 0.1% Tween-20 (TBS-T). The membranes were incubated overnight with primary antibody rabbit anti-MMP-2 (1:2000), rabbit anti-MMP-9 (1:2000), rabbit anti-iNOS (1:1000). After washing 3 times with TBS-T for every 5min, blots were incubated with peroxidase-conjugated anti-rabbit or anti-mouse secondary antibodies for 1h at RT. Finally, blots were rinsed and proteins were visualized using an ECL protein detection kit according to the manufacturer's instructions.

10. RT-PCR

Total RNA was isolated and purified with Trizol Reagent (Invitrogen, Carlsbad, CA, USA) according to the protocol recommended by the manufacturer. RNA was quantitated by measuring the absorbance at 260nm, and the ratio was 1.8 or higher. cDNA synthesis of mRNA was carried out by reverse transcription (RT). Normalization of the samples was accomplished using the reverse transcriptase-polymerase chain reaction (RT-PCR). PCR amplification for MMP-2 was performed at 94℃ for 30sec, 53℃ for 30sec and 72℃ for 30sec for 35 cycles. PCR amplification for MMP-9 was performed at

94℃ for 30sec, 59℃ for 30 sec and 72℃ for 30sec for 30 cycles. And the PCR amplification for iNOS was performed 94℃ for 30 sec, 57.5℃ for 30 sec and 72℃ for 30sec for 30 cycles. PCR amplification for caspase-3 was performed at 94℃ for 30 sec, 55℃ for 30sec and 70℃ for 30sec for 30 cycles. The sequences of the specific primers were as follows: sense, 5'- GAG TTG GCA GTG CAA TAC CT -3', and antisense, 5'- GCC GTC CTT CTC AAA GTT GT -3' for MMP-2; sense 5'- TTA CCA GCG CCA GCC GAC TTT TG -3' antisense, 5'- CGT CGT CGT CGA AAT GGG CAT C -3' for MMP-9; sense, 5'- GGA GCT GGA CTG TGG CAT TGA -3', and antisense, 5'- CAG TTC TTT CGT GAG CAT GGA -3' for caspase-3; sense, 5'- TTT GAT GTG CTG CCT CTG GT -3' and antisense, 5'- CAT TCT GCT TCT GGA AAC TAA GG -3' for iNOS; sense, 5'- ATG TCG TGG AGT CTA CTG GT -3' and antisense, 5'- TGG CAT GGA CTG TGG TG -3' for GAPDH. The PCR products were separated by electrophoresis in 1.5% agarose gel with ethidium bromide.

11. Statistical analysis

Results are presented as Mean ± SD. At least three different experiments were performed in separate cell preparation, duplicate or triplicate determination were performed in each experiments. One-way ANOVA followed by Student's t-test was used as indicated in order to examine the statistical significance; p-values less than 0.001 were considered significant.

III. RESULTS

Retroviral vector containing arginine decarboxylase (ADC) transfected to primary cultured astrocytes

It constructed the retroviral vector containing human ADC cDNA and established a transfection of ADC gene into the astrocytes by the retroviral packaging cell line PT67. The cell line transfected with a stably high expression of ADC and this was identified in RNA level (Fig 1).

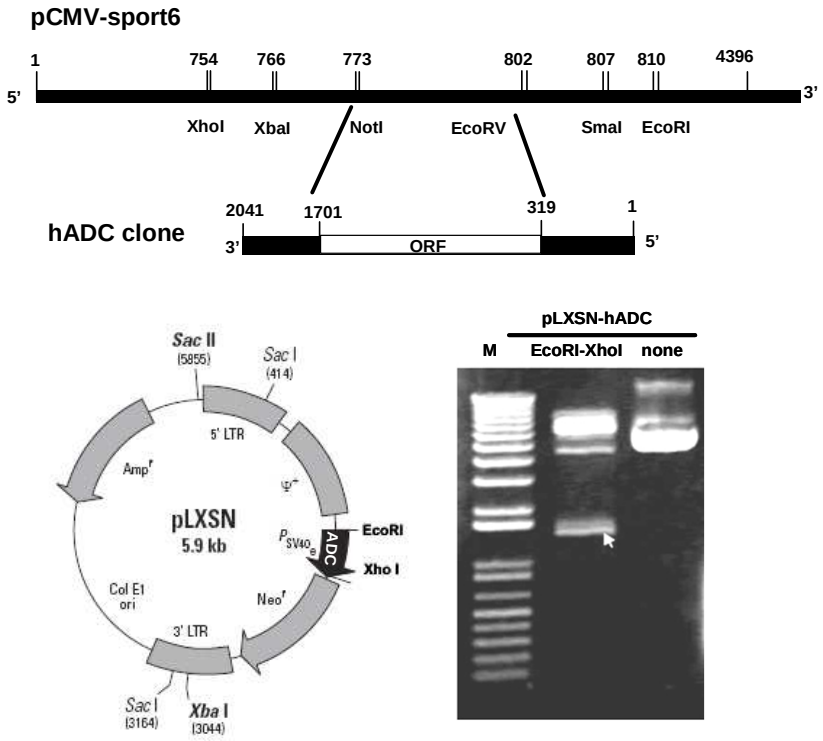


Figure 1. Construction of recombinant retrovirus vector which contain ADC gene
This construction is the human ADC clone obtained from hADC-pCMV-SPORT6. And EcoR I and Xho I were used for the analysis of excision recombinant in retroviral vector, pLXSN.

Expression of ADC genes in PT67 cells and primary astrocytes

The retro-X expression system (clontech K1060, USA) which was efficient to deliver in vitro the target gene into dividing mammalian cells was used. The human ADC full length cDNA (GenBank accession number AY325129) was amplified by PCR and recombined retro-pLXSN vector cloning reaction. The ADC-pLXSN/V5 expression plasmids were transfected into the retroviral packaging cell line PT67 using Lipofectamine 2000. Producing of ADC recombinant DNA construction, design of human ADC primer for verifying a transfection, amplification of the ADC transcripts by RT-PCR. It was performed using primers specific for ADC gene. 245 bp fragment of the ADC gene was seen in ADC infected PT67 cells and ADC infected astrocytes (Fig 2).

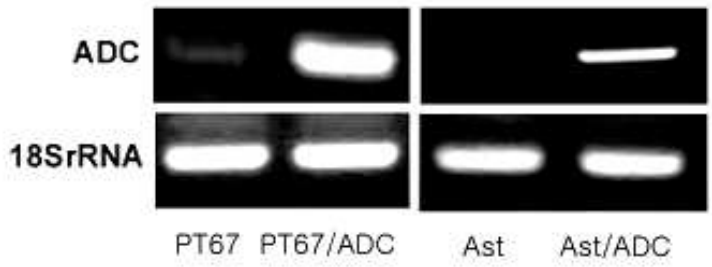


Figure 2. The construction and generation of retrovirus containing ADC gene
BD Retro-X universal packaging system directing expression of the ADC genes were used to infect primary astrocytes. Astrocytes expressing the viral genes were selected in G418. RT-PCR was performed using primers specific for ADC gene. 245 bp fragment of the ADC gene was seen in ADC infected PT67 cells which are retrovirus package cells, and ADC infected astrocytes.

Immunocytochemical analysis of ADC in primary cultured astrocytes

The expression of endogenous ADC in primary cultured astrocytes was detected by immuocytochemistry using ADC antibody. It is verified that ADC gene was overexpressed in ADC infected astrocytes (Fig 3). This experiment was repeated in three different preparation of astrocytes with similar findings.

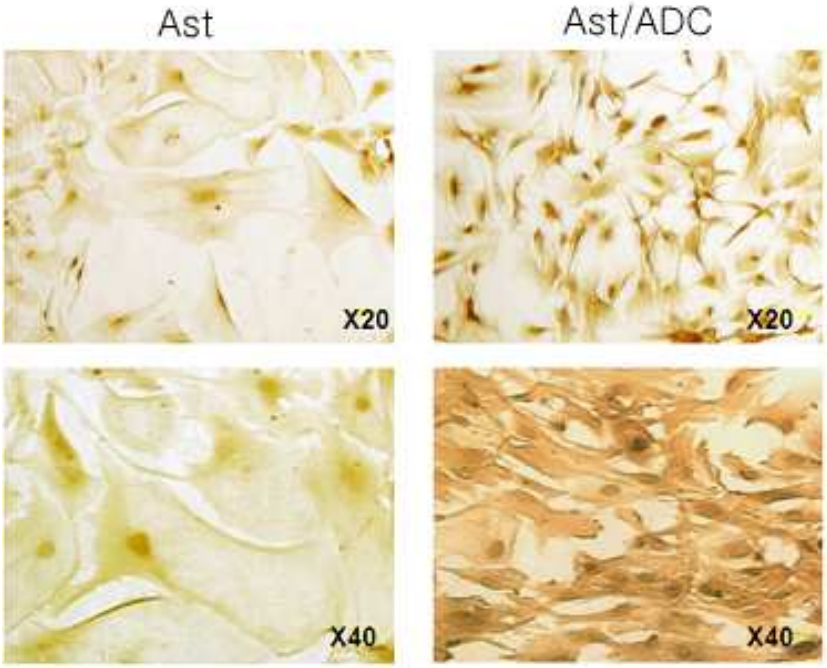
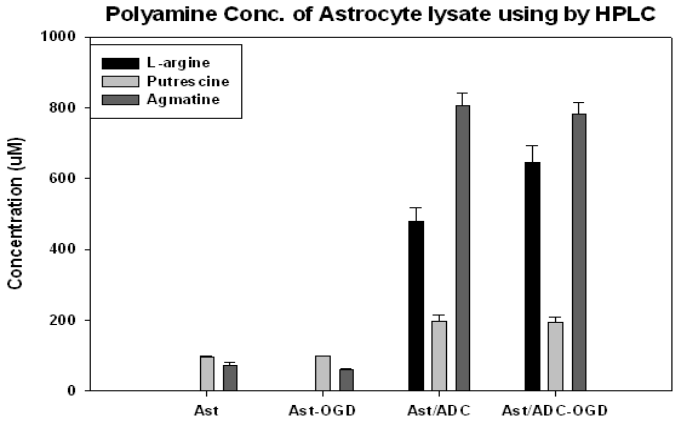


Figure 3. Immunocytochemical staining against ADC in the infected astrocytes The astrocyte were cultured from cortex of mouse brain. Cells were grown in culture plate. Confluent cells were replated by trypsinisation and used in experiments at passage2. Astrocytes were stained immunocytochemically with polyclonal ADC antibodies. (A , B): uninfected astrocytes, (C , D): astrocytes infected by ADC retovirus. Ast: primary cultured astrocytes, Ast-OGD: astrocytes injured by OGD, Ast/ADC: astrocytes expressing ADC gene, Ast/ADC-OGD: astrocytes expressing ADC gene under OGD injury.

The amount of agmatine generated endogenously were measured by HPLC in the astrocytes expressing ADC gene

The amount of agmatine in control and transfected cells was measured by HPLC. Agmatine can be produced by the arginine decarboxylase in astrocyte of mouse brain. It is verified that not only ADC was overexpressed but also agmatine was increased in astrocytes (Fig 4).



Folding ratio	Agmatine	Putrescine
Ast	1	1
Ast-OGD	0.857258	1.007242
Ast/ADC	11.27664	2.036347
Ast/ADC-OGD	10.9508	2.00685

Figure 4. The level of agmatine in the astrocytes expressing ADC gene
Agmatine levels were measured by high-pressure liquid chromatography (HPLC). The samples was mixed with *o*-pthalaldehyde (OPA) and injected into the HPLC system with column and detected by fluorescence detector. Ast: primary cultured astrocytes, Ast-OGD: astrocytes injured by OGD, Ast/ADC: astrocytes expressing ADC gene, Ast/ADC-OGD: astrocytes expressing ADC gene under OGD injury.

Protective effect of agmatine generated endogenously in the primary cultured astrocytes expressing ADC gene after oxygen glucose deprivation injury

The neuroprotective effects of endogenous agmatine against hypoxia-induced damage to astrocytes was studied using Hoechst33258 and propidium iodide double staining. To investigate the protective effect of ADC gene expression on OGD injury, primary cultured astrocytes were subjected to OGD 4hours. The effect of ADC gene expression was analysed by Hoechst(Blue)-PI(red) nuclear staining (Fig 5). The control astrocytes exhibited confluent Hoechst positive cells. Apoptosis of astrocytes was observed in injured group, but the degree of cell death decreased in ADC infected astrocytes(Ast/ADC) compared to uninfected astrocytes(Ast). Exposure to hypoxia for 4 hours resulted in a significant loss of Hoechst positive cells and many PI positive cells. This decreases of apoptosis in the astrocytes may explain the protective effect of endogenous agmatine after OGD injury.

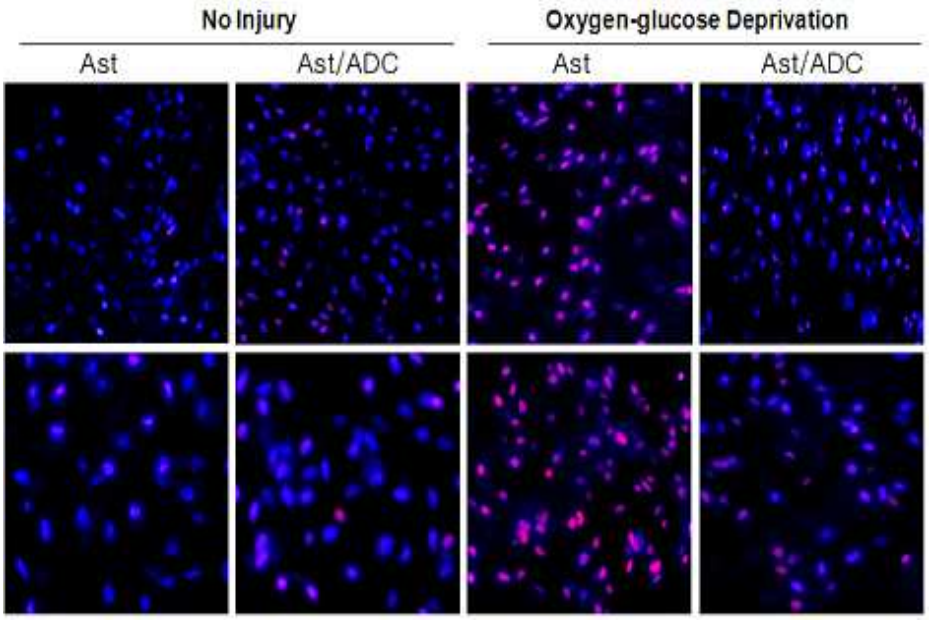


Figure 5. Photographs showing astrocyte stained with Hoechst propidium iodide Primary cultured astrocytes expressing ADC gene were stained with Hoechst (Blue)-propium iodide (red) after OGD injury. The large diffusely fluorescent nuclei stained by Hoechst dye were in intact cells by PI exclusion criteria. Smaller, pycnotic nuclei were present in cells which had already lysed as determined by PI staining.

Effect of endogenous agmatine on the astrocyte at 4hrs after ischemia-like injury

To investigate the protective effect of endogenous agmatine on OGD injury, primary cultured astrocytes were transferred to anaerobic chamber for 4hrs after infection or uninfection. As shown in figure 6, exposure to a hypoxic environment for 2 , 4 , 10 hours significantly increased release of lactate dehydrogenase(LDH). The effect of endogenous agmatine on LDH release into the culture medium. In

OGD-R subjected cells, LDH level in the medium was gradually increased. However, treatment with ADC gene caused an attenuation of LDH release in astrocytes under OGD. ADC infected astrocytes group did not show significantly amounts of LDH release. LDH release (%) was measured to know the degree of cell death under four different reperfusion times.

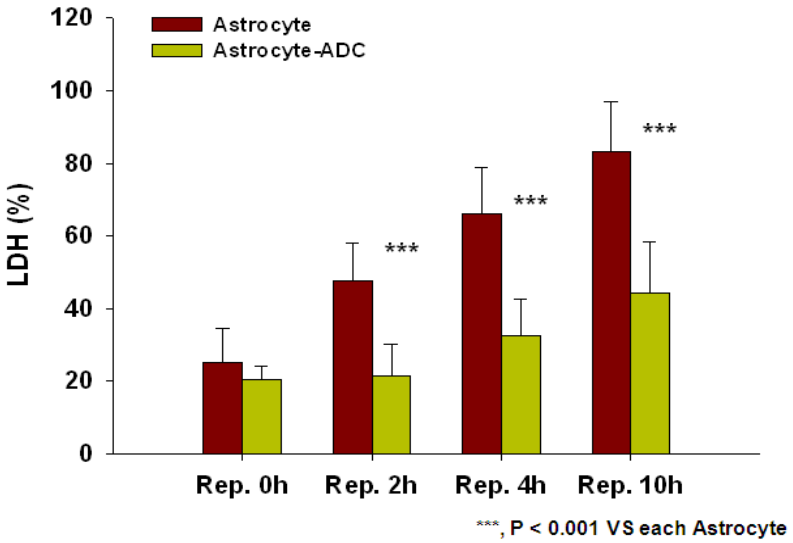


Figure 6. Protective effect of endogenous agmatine on the primary cultured astrocytes after ischemic injury LDH release(%) was assayed for relative total cellular death. Lactate dehydrogenase (LDH) activity released into the bathing medium by uninfected cells and Ast/ADC was measured. The Ast/ADC were significantly less injured than uninfected cells (*** $p < 0.001$ vs. each astrocyte). Data are expressed as Mean±SD.

Immunocytochemical staining and immunoblotting of caspase3 in Astrocyte after OGD injury

Immunofluorescence staining against caspase-3 was performed in uninfected astrocyte and Ast/ADC after OGD injury. Also, immunoblotting study was done to investigate whether the expression of caspase3 is related to the decrease of cell death. The number of caspase 3 positive cells were increased after OGD, but decreased in Ast/ADC (Fig 7).

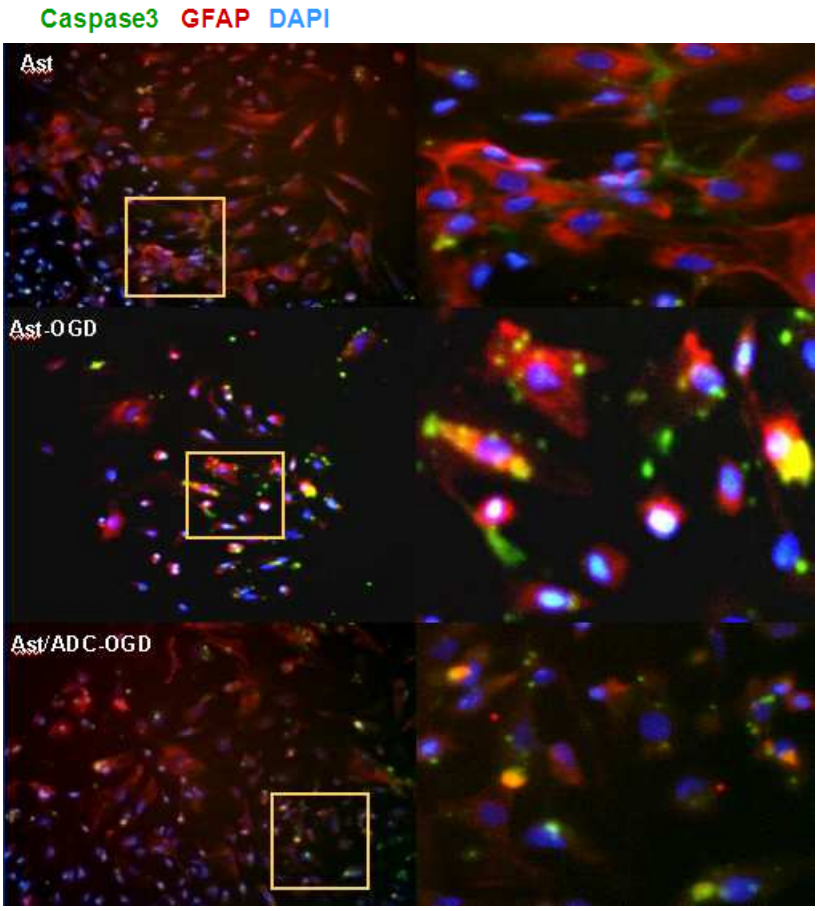


Figure 7. Identification of caspase-3 immunoreactive cells in the astrocyte

The primary cultured astrocytes were grown in culture plate. Confluent astrocyte were replated by trypsinisation and used in experiments at passage 2. Cells were maintained in the hypoxia chamber for 4h and returned to the incubator. Glucose was added to the culture medium to a final concentration of 5.5mM for 4h under 5% CO_2/95% air condition. Normal astrocyte were not exposed to OGD. This experiment was repeated in three different preparation of astrocytes with similar findings.

The mRNA expression of caspase 3 in astrocytes expressing ADC genes after OGD injury

In order to determine whether endogenous agmatine regulates the mRNA expression of caspase-3 in cultured astrocytes after OGD injury. As shown in Fig 8, the expression of caspase-3 was decreased in AST/ADC-OGD group compared to the AST-OGD. Accordingly, RT-PCR analysis demonstrated that endogenous agmatine also modulates caspase-3 expression at the mRNA level (Fig 8). The PCR method is to identify and quantitate the expression of gene levels will be a valuable tool in evaluating the regulation of the synthesis of agmatine in astrocytes.

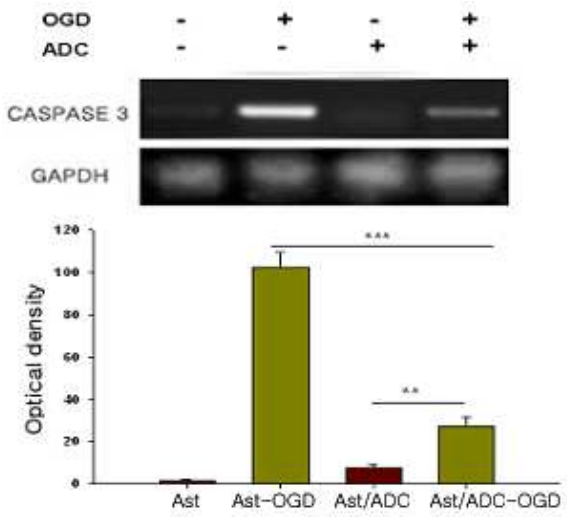
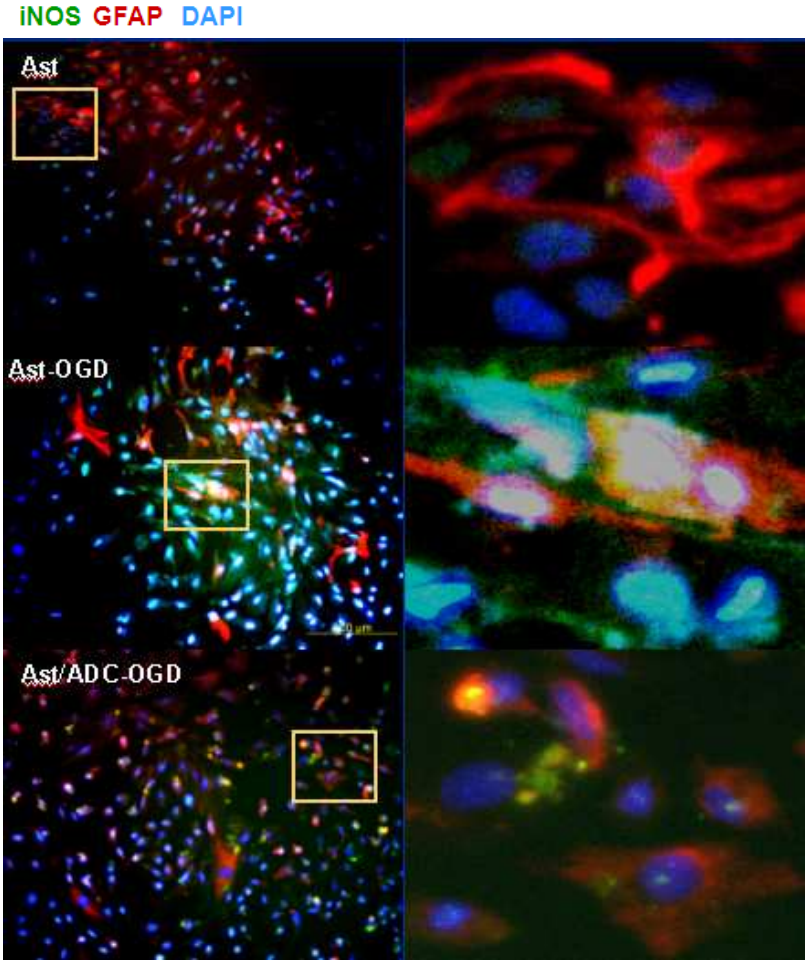


Figure 8. The effect of astrocyte expressing ADC gene on mRNA levels of caspase-3 Endogenous agmatine regulates caspase3 expression at the mRNA levels. Total RNA was used for cDNA synthesis using reverse transcriptase. PCR was performed for cDNA using specific primers. Lane:1, Astrocyte; 2, Ast-OGD; 3, Ast/ADC; 4, Ast/ADC-OGD. The simultaneous PCR was performed using primers for GAPDH in these cDNA samples to normalize the change. Relative optical densities (ROD) of caspase-3 were expressed as the band density of each group (N=3) compared to the normal control (**, $p < 0.01$; *** $p < 0.001$ vs. ADC uninfected astrocyte). Data are expressed as Mean±SD.

Immunocytochemical staining and immunoblotting of iNOS in Astrocytes-ADC after OGD injury

In order to verify whether endogenous agmatine had protective effects on hypoxia-induced apoptotic death of astroctes, further analysis using immunofluorescence staining and immunoblotting study were performed. As shown in Fig 9, the expression of iNOS were increased at protein level in AST-OGD group, and decreased in AST/ADC-OGD group. In similar to iNOS immunocytochemical result, the number of iNOS positive cells was increased in AST-OGD group, respectively, and astrocyte expressing ADC genes decreased the number of iNOS immunopositive cells significantly. Accordingly we confirmed that the expression of iNOS was decreased by astrocyte expressing ADC gene following OGD injury.


iNOS GFAP DAPI
Ast
Ast-OGD
Ast/ADC-OGD

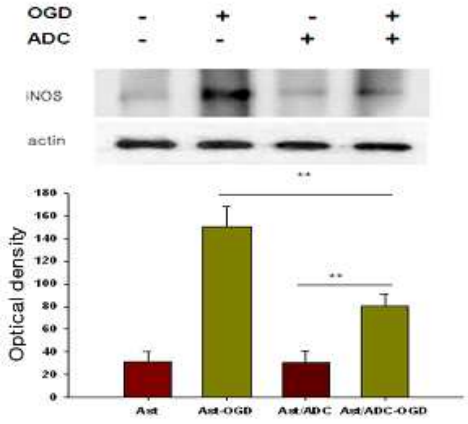

OGD
ADC
iNOS
actin
Optical density
Ast
Ast-OGD
Ast/ADC
Ast/ADC-OGD

Figure 9. Identification of iNOS immunoreactive cells in the astrocytes The primary cultured astrocytes were grown in culture plate. Confluent astrocyte were replated by trypsinisation and used in experiments at passage 2. Cells were maintained in the hypoxia chamber for 4h and returned to the incubator. Glucose was added to the culture medium to a final concentration of 5.5mM for 4h under 5% CO_2/95% air condition. Normal astrocyte were not exposed to OGD. The expression of iNOS was decreased by ADC overexpression after OGD injury. This experiment was repeated in three different preparation of astrocytes with similar findings (**, $p < 0.01$ vs. ADC uninfected astrocyte). Data are expressed as Mean±SD.

The mRNA expression of iNOS in astrocytes expressing ADC genes after OGD injury

The next question was to determined whether endogenous agmatine is regulate the expression of iNOS in cultured astrocytes after OGD injury. As shown in Fig 10, the expression of iNOS was decreased in AST/ADC-OGD group compared to the AST-OGD. Similarly, RT-PCR analysis demonstrated that endogenous agmatine also modulates iNOS expression at the mRNA level. The PCR method is to identify and quantitate the expression of gene levels will be a valuable tool in evaluating the regulation of the synthesis of agmatine in astrocytes.

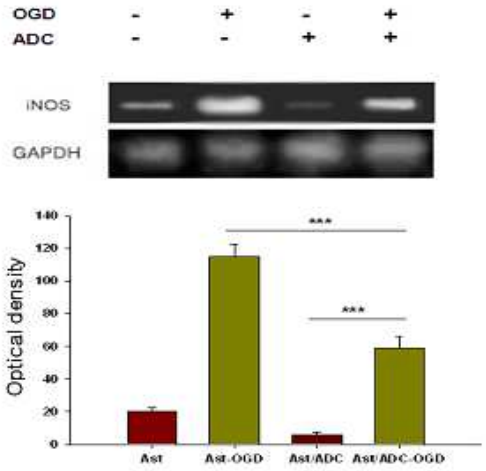
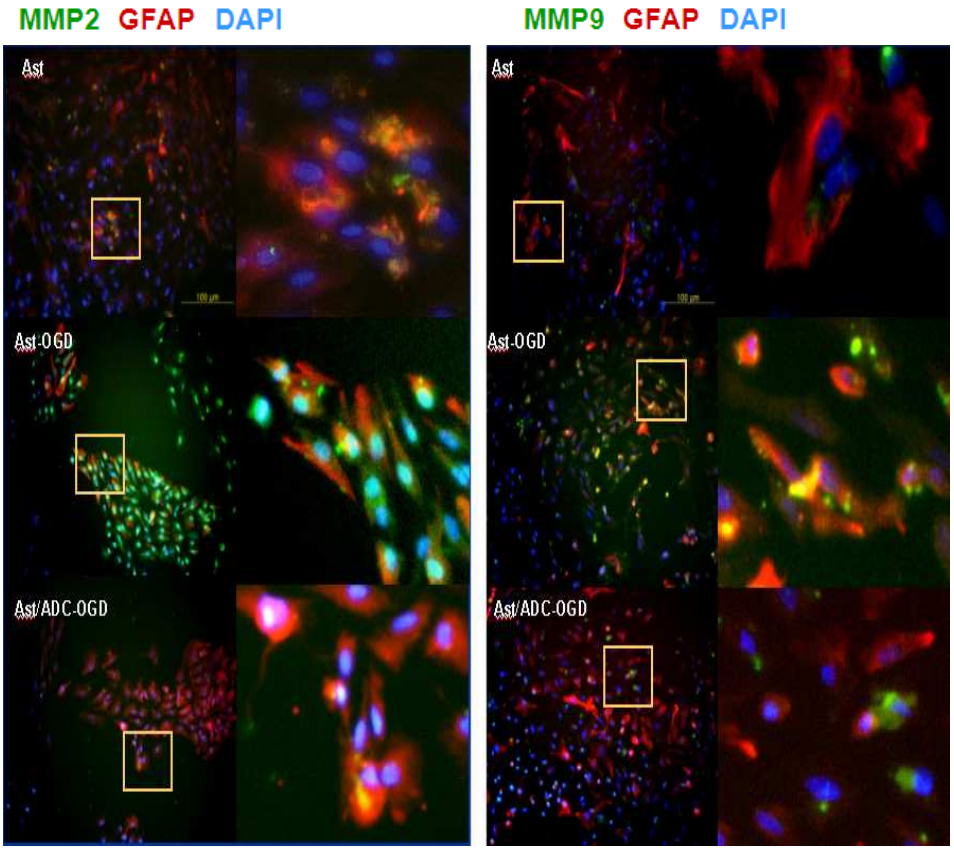


Figure 10. The effect of astrocyte expressing ADC gene on mRNA levels of iNOS Endogenous agmatine regulates iNOS expression at the mRNA levels. Total RNA was used for cDNA synthesis using reverse transcriptase. PCR was performed for cDNA using specific primers. Lane:1, Astrocyte; 2, Ast-OGD; 3, Ast/ADC; 4, Ast/ADC-OGD. The simultaneous PCR was performed using primers for GAPDH in these cDNA samples to normalize the change. Relative optical densities (ROD) of iNOS were expressed as the band density of each group compared to the normal control (***, $p < 0.001$ vs. ADC uninfected astrocyte). Data are expressed as Mean±SD.

Immunocytochemical staining and immunoblotting of MMP2 and MMP9 in ADC-astrocytes after OGD injury

Immunofluorescence staining and western blot against MMP2 and MMP9 were performed in astrocytes and ADC infected astrocytes. In order to investigate whether the expression of MMP2 and MMP9 is related to the decrease of cell death, immunofluorescence stainnng and immunoblotting were performed. The expression of MMP2 and MMP9 protein was decreased by ADC infection. Accordingly, we confirmed that the expression of MMP2 and MMP9 was decreased in the astrocyte expressing ADC gene after OGD injury. In similar to MMP2 and MMP9 immunocytochemical result, the number of MMP2 and MMP9 positive cells was decreased in AST/ADC-OGD group compared to the AST-OGD (Fig 11).


MMP2 GFAP DAPI
MMP9 GFAP DAPI
Ast
Ast-OGD
Ast/ADC-OGD
Ast
Ast-OGD
Ast/ADC-OGD

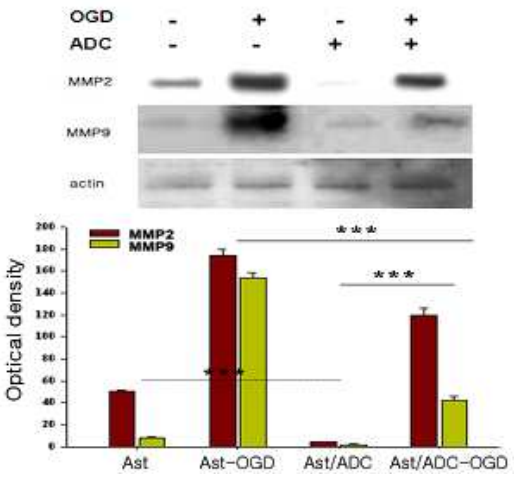

OGD - + - +
ADC - - + +
MMP2
MMP9
actin
MMP2
MMP9
Optical density
Ast
Ast-OGD
Ast/ADC
Ast/ADC-OGD

Figure 11. Immunocytochemical staining and immunoblotting of MMP2 and MMP9 in ADC-astrocytes after OGD injury The primary cultured astrocytes were grown in culture plate. Confluent astrocyte were replated by trypsinisation and used in experiments at passage 2. Cells were maintained in the hypoxia chamber for 4h and returned to the incubator. Glucose was added to the culture medium to a final concentration of 5.5mM for 4h under 5% CO_2/95% air condition. Normal astrocyte were not exposed to OGD. This experiment was repeated in three different preparation of astrocytes with similar findings (***, $p < 0.001$ vs. ADC uninfected astrocyte). Data are expressed as Mean±SD.

The mRNA expression of MMP2 and MMP9 in ADC-astrocytes after OGD injury

In order to verify whether endogenous agmatine regulates the mRNA level of MMP2 and MMP9 in cultured astrocytes after OGD injury. As shown in Fig 12, the expression of MMP2 and MMP9 was decreased in AST/ADC-OGD group compared to the AST-OGD. And the mRNA expression of MMP2 and MMP9 was decresed in ADC infected astrocytes compared to normal astrocytes both with and withought OGD injury. Therefore, RT-PCR analysis demonstrated that endogenous agmatine also modulates MMP2 and MMP9 expression at the mRNA level. The PCR method is to identify and quantitate the expression of gene levels will be a valuable tool in evaluating the regulation of the synthesis of agmatine in astrocytes.

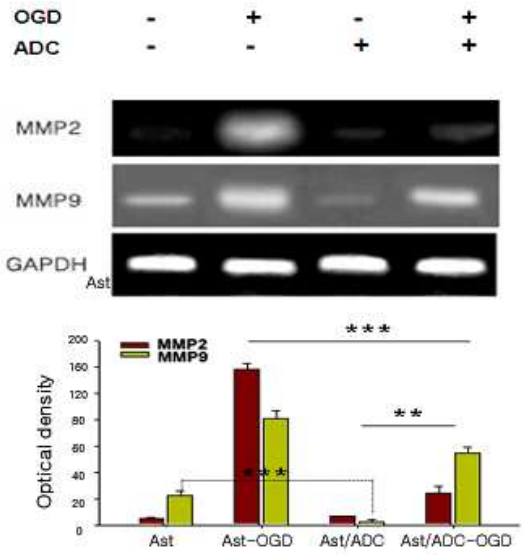


Figure 12. The effect of astrocyte expressing ADC gene on mRNA levels of MMP2 and MMP9 Endogenous agmatine regulates MMP2 and MMP9 expression at the mRNA levels. Total RNA was used for cDNA synthesis using reverse transcriptase. PCR was performed for cDNA using specific primers. Lane:1, Astrocyte; 2, Ast-OGD; 3, Ast/ADC; 4, Ast/ADC-OGD. The simultaneous PCR was performed using primers for GAPDH in these cDNA samples to normalize the change. Relative optical densities (ROD) of MMP2 and MMP9 were expressed as the band density of each group compared to the normal control (**, $p < 0.01$; ***, $p < 0.001$ vs. ADC uninfected astrocyte). Data are expressed as Mean±SD.

IV. DISCUSSION

Agmatine has been shown to have several biologic actions in brain and in the periphery.[8-14] Of particular interest among the actions of agmatine is its ability to offer protection against ischemic injury and chronic neuropathic pain.[15-18] Following previously reports, agmatine decreased infarct sizes in middle cerebral artery occulusion mouse model, and promotes survival in neurons exposed to OGD[30,36,50].

Endogenous agmatine, produced by ADC genes, have many possible pathway to inhibit the cell death, NMDA channel blocker, competitive inhibitor of NOS, and so on[26]. It has been reported that this protection by agmatine may associated with decreased iNOS and MMPs expression.

Thus, one of our notable findings is that agmatine synthesized endogenously through overexpression of ADC genes is neuroprotctive material in ischemic injury. There are several possible mechanism of endogenous agmatine induced neuroprotection. It is focused on the regulation of iNOS and MMPs.

Here, it is characterize neuroprotective effects of endogenous agmatine in astrocytes exposed to OGD-R. It is needed to confirm whether the astrocytes is infected by ADC retroviral vector system. So, the overexpression of ADC genes in astrocytes was confirmed by RT-PCR, immuocytochemistry. Subsequently, the amount of endogenous agmatine synthesized by overexpressed ADC genes was measured by HPLC. It is verified that not only ADC was overexpressed but also agmatine was increased in astrocytes. It has demonstrated that incubation of astrocytes with ADC significantly reduces the

cell death by OGD-R as measured by the accumulation of LDH in the medium. This reduction in LDH accumulation is consistent with lower PI-stained nuclei in ADC treated astrocytes as measured by the Hoechst-PI nuclear staining.

Recent studies have shown that agmatine has a neuroprotective effect in *in vivo* and *in vitro* against ischemic injury.[15,18,30] It has been reported that this protection by agmatine is associated with decreased NOS activity and expression. NO is synthesized by three different NOS isoforms and is thought to function as both a neurotransmitter and a neurotoxin[33,35]. Astrocytes produce NO mainly from iNOS. In this study, it was hypothesized that endogenous agmatine could suppress cell death through inhibiting NOS activity. It has been demonstrated that the expression of iNOS were increased at protein level in AST-OGD group, and decreased in AST/ADC-OGD group. In similar to iNOS immunocytochemical result, the number of iNOS positive cells were increased in AST-OGD group, respectively, and astrocyte expressing ADC genes decreased the number of iNOS immunopositive cells significantly. As a result of that, the expression of iNOS was reduced by astrocyte expressing ADC gene. And it is demonstrated that endogenous agmatine reduced the production of iNOS. Endogenous agmatine may act as a neuroprotector by suppression of iNOS induction.

Following ischemia, there is a loss of microvascular integrity which is manifested by major alterations in vascular permeability. As a result, the vascular extracellular matrix is lost, which is a constituent of the basal lamina, which plays a critical role in maintaining the integrity of the BBB on the astrocytes cell wall by providing structural supports. Matrix metalloproteinases

(MMPs) degrade the basement membrane of brain vessels to promote cell death and tissue injury[56,57]. In the present study, it is investigated the production of MMP-2 and MMP-9 by CNS microvascular endothelium, and demonstrated that both MMP-2 and MMP-9 are up-regulated by these cells *in vitro* following ischemia insults[25,42,43]. It is found that astrocytes expressing ADC genes led to MMP-2 and MMP-9 reduction under ischemic injury. To investigate the modulation of matrix metalloproteinases expression by endogenous agmatine in protein and mRNA level, it is performed immunocytochemistry, western blot and RT-PCR. The result showed that immunopositive cells were significantly reduced in agmatine endogenously administrated group. It also observed in the western blot analysis that astrocytes expressing ADC gene attenuated the expression of the MMP-2 and MMP-9 induced by ischemic injury.

One of this study notable findings is that agmatine synthesized endogenously through overexpression of ADC genes decrease iNOS and MMPs expression in astrocytes under OGD injury. This decreases of iNOS and MMPs expression may explain the protective mechanism of agmatine after ischemia.

In conclusion, it has demonstrated that astrocyte expressing ADC gene representated neuroprotective effects is related to the iNOS, caspase-3 and MMPs gene level. It may suggest that endogenous agmatine in the astrocyte expressing ADC gene may play a permissive role through reduced the degree of cell death in neuronal pathology induced by OGD stress. Accordingly, endogenous agmatine may act as a neuroprotector.

V. CONCLUSION

This present study showed the role of endogenous agmatine on cell death induced by OGD injury. These results have demonstrated following conclusion.

1. Agmatine administered endogenously through overexpression of ADC which is a synthesizing enzyme was produced in astrocytes.

2. Neuroprotective effects of agmatine generated endogenously may involve inhibition of iNOS.

3. The expression of MMP2 and MMP9 were suppressed by endogenously administered agmatine after OGD injury in astrocytes.

4. Endogenous agmatine, produced by ADC gene, may lead to a novel therapeutic material to reduce cell death related to hypoxia.

Taken together, these data would find a way that endogenous agmatine levels in the astrocyte expressing ADC gene may play a permissive role through reduced the degree of cell death in neuronal pathology induced by OGD stress. Agmatine synthesized endogenously through overexpression of ADC genes decrease iNOS and MMPs expression in astrocytes from OGD injury and this decreases may explain the protective mechanism of endogenous agmatine after ischemia.

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<ABSTRACT(IN KOREAN)>

허혈 손상시 ADC 과발현 별아교세포의 신경보호 효과

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손 미 란

아그마틴은 L-알기닌의 탈탄산화에 의해 생기는 일차 아민으로써, 포유류의 뇌에서 생성되는 내재성 클로니딘 대체물질이다. 아그마틴은 NOS의 길항제로서 중추신경계에서 신경보호물질과 신경보호매개물로써의 능력을 지니고 있다. 이전 연구 결과에서 아그마틴을 실험동물모델에 주입 하였을 때 허혈 손상으로부터 뇌의 손상과 부종을 줄여준다는 연구결과를 확인한 바 있다. 본 연구에서는 뇌혈관질환에서 이미 신경세포사멸의 방어물질로 효과가 규명되고 있는 아그마틴을 세포내 자가 생성해내는 ADC 유전자를 이용하여 아그마틴의 자가생성을 유도함으로서 내재성 아그마틴의 신경세포 방

어 기전에 대해 조사하였다.

생체 내에서 세포의 생존 및 내성을 강화시키는 물질인 아그마틴을 생성하는 알기닌 탈탄산효소 유전자를 함유한 바이러스 벡터를 제조하였고, 레트로 바이러스 시스템을 통해 ADC 유전자를 생후 1~3일된 흰 쥐의 신경세포에 도입하여 ADC 과다발현 세포를 제작하였다. 이렇게 도입된 ADC 유전자는 ADC 항체를 이용한 면역염색과 RT-PCR 기법으로 별아교세포 내에 도입된 ADC 유전자를 확인하였다. 도입된 ADC 유전자에 의해 과발현된 아그마틴은 고성능 액체 크로마토크래피법인 HPLC를 통하여 세포내에서 정상적으로 자가생성된 아그마틴의 양을 정량 분석하였다.

또한, 별아교세포에 산소와 글루코즈의 차단을 통한 세포내 허혈 손상을 주었을때 과발현 된 아그마틴이 신경세포를 보호하는 기전을 연구하였다. 자가 생성된 아그마틴으로 인해 뇌 허혈 손상 시 MMPs의 발현양의 조절을 관찰하였으며 그 기전에 대해 연구하였다. 연구 결과 세포허혈 손상 시 내재성 아그마틴에 의해 MMP-2 와 MMP-9 의 발현양이 감소되는 것을 확인할 수 있었으며 이는 단백질 level에서 와 마찬가지로 RNA level 에서도 유사한 결과를 나타내었다. 이러한 세포보호효과는 아그마틴이 NOS의 발현을 조절하는 것과 연관되어 있다는 것과 NOS 의 발현양의 감소는 신경세포 보호에 영향을 미친다는 보고에 따라 본 연구에서도 iNOS 와 세포사관련 물질인 caspase-3 등의 물질을 함께 확인하였다. 신경세포에 허혈 손상을 준 후 아그마틴에 의한 신경보호 작용이 iNOS의 발현과 어떠한 연관성이 있는지 조사한 결과 iNOS 면역염색법에서 세포 손상시 ADC 유전자가 도입되지 않은 별아교세포에서는 세포사멸이 현저히 관찰되나 ADC 가 도입된 별아교세포에서는 iNOS 양성반응 세포의 수가 증가한 것을 확인하였다. 세포 내에서 자가 생성된 아그마틴에의해 iNOS, caspase3, MMP2 와 MMP9 의 발현양이 조절되는 것을 여러 가지 분자생물학적 실험기법을 통해 확인할 수 있었다.

이를 통해 ADC 유전자의 과발현에 의한 아그마틴의 세포내 자가생성됨은 별아교세포의 세포 손상모델에서 이러한 세포사 관련 물질을 줄여 줌으로써 허혈 손상시 신경세포 보호 효과를 나타냄을 확인 할 수 있었다. 따라서 유전자 치료 물질로서의 알기닌 탈탄산효소가 신경 세포 보호 효과를 내는데 중요한 물질임을 알수 있다.

핵심어 : 알기닌 탈탄산효소, 아그마틴, 별아교세포, 허혈, 신경보호