Role of Angiotensin II Type 2 Receptor and VEGF-D in Cerulein-Induced Inflammation of Pancreatic Acinar Cells

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Role of Angiotensin II Type 2 Receptor and VEGF-D in Cerulein-Induced Inflammation of Pancreatic Acinar Cells

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The doctoral dissertation submitted to the Department of Medical Science, the Graduate School of Yonsei University in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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“실패를 걱정하지 말고, 먼저 부지런히 목표를 향하여 노력하라. 노력한 만큼 보상을 받을 것이다. 쉬운 길, 편안한 길로 가는 사람은 성공의 묘미를 못 느낀다. 어려움 없이 성취되는 것은 하나도 없다. -노만 V. 필.”

대학원 박사과정에 들어와서 연구실 책꽂이에 꽂혀 있던 이우주 선생님의 자서전 한 권이 오늘의 저를 있게 해준 삶의 지표가 된 듯 합니다. 시련을 실패로 착각하거나 실패가 두려워 도전하기를 꺼려하는 것은 어쩌면 나약한 인간의 본성일지 모릅니다. 삶과 실험이 힘들 때마다, 제 자신의 한계에 부딪쳐 도망치고 싶을 때마다, 제게 언제나 희망이라는 단어와 최선을 다하면 이루지 못할게 없다는 신념을 가지게 해준 이 책이 제게 제 자신과의 싸움에서 조금이나마 이겨 나갈 수 있는 힘이 된 것 같습니다.

구체적인 목표가 있는 사람은, 조금 돌아가더라도 확고한 신념과 목표가 있다면 그 꿈을 이룰 수 있다고 생각합니다. 그러나 그 꿈을 이루기 위해서는 그 길을 올바르게 갈 수 있도록 인도해 주는 안내자와 나침반이 필요하다고 생각합니다. 그런 면에서 저는 신의 은총으로 훌륭한 안내자를 만나서 너무나도 운이 좋은 사람입니다. 박사과정 5년 동안, 너무나도 부족한 저를 언제나 믿어주시고 기회를 주신 김혜영 교수님, 약리학 교실원으로서의 자부심을 심어주신 김경환 교수님께 먼저 머리숙여 마음깊이 감사의 인사를 드리고 싶습니다. 가는 길이 힘들어 포기하고 싶을 때도, 제 자신을 믿지 못해 모든 것이 실패라고 생각했을 때도, 저를

감싸주시고 제게 올바른 길로 갈 수 있는 지혜와 자세를 가르쳐 주신 교수님께 더 좋은 결과를 보여 드리지 못한 것 같아 너무나 송구스럽습니다. 하지만 두 분 교수님의 지도가 있었기에 어떠한 역경에서도 꿋꿋히 헤쳐나갈 수 있는 지혜와 용기를 가지고 헤쳐 나갈 수 있게 되었다고 말씀드리고 싶습니다.

저의 연구에 언제나 소홀함을 지적해주시고 격려하여주신 안영수 교수님, 삶에 있어서의 목표와 용기를 언제나 강조해주신 김동구 교수님, 저의 연구에 부족한 부분을 지적하여주시고 좋은 아이디어를 주신 이민구 교수님, 늘 미소로 대해주시고 항상 좋은 말씀해주시는 박경수 교수님, 진심으로 감사드립니다.

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마지막으로 공부하는 남편을 뒷바라지하며 고생하는 저의 소중한 인연 황수양씨에게 감사드립니다. 제 아내를 만나지 못했다면 지금의 제가 없었을 것입니다. 힘들 때에도, 항상 행복을 더 많이 가져다주는 소중한 딸 경민이에게 고맙다는 말을 전합니다.

이제 마무리할 대학원 생활에 아직도 많은 미련과, 좀 더 잘할 수 있었을 것이라는 후회가 남습니다. 이제 졸업이라는 하나의 계단을 올라왔습니다. 아직도 제가 올라갈 많은 계단이 남아 있습니다. 저를 아껴주시고 사랑해 주신 모든 분들께 감사 인사를 드리며, 이제 또 하나의 계단을 오르기 위해 또 노력하고자 합니다.

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(Directed by Professor Kyung Hwan KIM)

Cerulein-induced pancreatitis model is similar to human acute edematous pancreatitis. Their common characteristics include dysregulation of the digestive enzyme production, aberrant cytoplasmic vacuolization, death of acinar cells, edema formation, and infiltration of inflammatory cells into the pancreas.

The present study was designed to elucidate the role(s) of VEGF-D and angiotensin II type 2 receptor in inflammatory signaling in cerulein-stimulated rat pancreatic AR42J cells. Stimulation of AR42J cells by cerulein caused differential expression of several membrane proteins. Among these proteins, level of VEGF-D was increased by cerulein treatment. Angiotensin II type 2 receptor, another renin-angiotensin system component, is localized and associated with cerulein-induced inflammation in pancreatic acinar cells. In this study, it was shown that angiotensin II type 2 receptor interacts with VEGF-D.

Cytokine expression was inhibited by treatment with ERK1/2 or MEK1

antagonists as well as by transfection with dominant negative genes such as IκB mutant, RasN-17 (for Ras) or TAM67 (for c-Jun) in cerulein-stimulated AR42J cells. This indicates that NF-κB, AP-1 and ERK1/2 are involved in cerulein-induced inflammation. Furthermore, cerulein-induced activation of NF-κB and AP-1 was inhibited by antagonist of ERK1/2. This suggests that activation of NF-κB as well as that of AP-1 is mediated by ERK signaling pathways.

Cerulein induced translocation of VEGF-D to the cell surface, and increased its interaction with angiotensin II type 2 receptor. Antisense-oligonucleotide for VEGF-D suppressed activation of NF-κB and AP-1 as well as IL-6 expression in cerulein-stimulated AR42J cells. This demonstrates that VEGF-D activates expression of cytokines through activation of AP-1 and NF-κB. Additionally, expression of cytokines was inhibited by angiotensin II type 2 receptor antagonist but not by VEGF receptor antagonist in cerulein-stimulated AR42J cells. In conclusion, VEGF-D mediates the inflammatory signaling of pancreatic acinar cells through interaction with angiotensin II type 2 receptor.

Key words: VEGF-D, Angiotensin II type 2 receptor, cerulein, acute pancreatitis

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I. INTRODUCTION

Acute pancreatitis is a multifactorial disease associated with the release of digestive enzymes into the pancreatic interstitium and to the systemic circulation[1-3]. The production and release of cytokines are increased in acute pancreatitis, which can ultimately lead to the deleterious local and systemic effects. Despite considerable progress in understanding pathophysiology of pancreatitis, the mechanisms of the development of this disease remain obscure. A number of animal models in experimental pancreatitis have been well developed. Administration of supramaximal dose of cerulein is one of the best characterized and widely studied experimental pancreatitis models. Supramaximal dose of cerulein results in dysregulation of the production and secretion of digestive enzymes. It also causes cytoplasmic vacuolization and death of acinar cells, edema formation, and infiltration of inflammatory cells

into the pancreas[4-6]. Cerulein induces reactive oxygen species (ROS) production, cytokine expression and apoptosis in rat pancreas and in pancreatic acinar cell line[6, 36-38]. However the pathogenic mechanism of pancreatitis has not been clarified. Over the past several years, evidences have accumulated on the involvement of inflammatory mediators in the development of pancreatitis[7, 8]. Systemic levels of interleukin (IL)-6 and IL-8 are increased in patients with acute pancreatitis and correlated with the severity of the disease. There is considerable evidence that pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α and IL-1β play a central role in acute pancreatitis and may mediate the systemic complication of acute pancreatitis[8, 16, 17]. IL-1β and TNF-α act as proximal mediators, and induce production of other mediators including IL-6 and IL-8[8]. The cellular mechanism of these inflammatory molecules involves activation of transcription factors such as NF-κB and AP-1[18]. Activation of NF-κB in the pancreas has recently been demonstrated in rat cerulein-pancreatitis. Inhibition of NF-κB activation resulted in a decrease in the expression of cytokines[16, 19, 20]. AP-1 as well as NF-κB represents early response transcriptional complexes essential for the gene expression of inflammatory molecules. MAP kinases are serine-threonine-directed kinases originally identified as being activated by mitogens. They are activated by a variety of stimuli including growth factors, cytokines, neurotransmitters, hormones, extracellular matrix molecules, and cell stress[21, 36, 37]. MAP kinases regulate a number of cellular processes including gene transcription, protein translation, metabolism, and the function of the cytoskeleton. Also, MAP kinases are involved in control of cell growth, differentiation, survival, and apoptosis. Currently, there are five independent MAP kinase cascades, but information

on their function in the pancreas and other differentiated cells is limited to the cascades leading to the extracellular signal-regulated kinase1/2 (ERK1/2), c-Jun-NH2-terminal kinases1/2 (JNK1/2), and p38 MAP kinase[22].

To understand early pathophysiologic mechanism of acute pancreatitis, differentially expressed membrane-proteins were analyzed in cerulein-stimulated AR42J cells[52]. Among these membrane-proteins, VEGF-D was increased by cerulein. VEGF family is involved in renin-angiotensin system components. It activates ERK1/2[23] and its human orthologue shares 84% identity. It was named VEGF-D because its primary sequence is most similar to VEGF-C. Both VEGF-C and VEGF-D are recognized by VEGF receptor-2 and VEGF receptor-3, which are present on endothelial cells[32-34].

Recently, it was reported that renin-angiotensin system components are present in isolated acinar cells as well as AR42J cell line[24, 26, 27]. Interestingly, pancreatic inflammatory conditions such as pancreatitis, hypoxia, and fibrosis can upregulate the renin-angiotensin system components in the pancreas, leading to further inflammation and tissue injury[23, 34]. This suggests that these may play an important role in the signal transduction of pancreatitis-induction[24]. Also, recently, it was reported that VEGF-D expression is related to ERK1/2 activation in several cells[25-27, 34] and the inflammation of these cells is induced by interaction of VEGF-D with angiotensin II type 2 receptor [28, 29]. However, the role of VEGF-D in signaling pathway of pancreatitis has not known yet. The present study was designed to elucidate whether VEGF-D is associated with angiotensin II type 2 receptor, and involved in cytokine expression in cerulein-stimulated AR42J cells. The roles of VEGF-D and angiotensin II type 2 receptor in inflammation of cerulein- stimulated pancreatic acinar cells were also investigated.

II. MATERIALS AND METHODS

1. Cell line

The rat pancreatic acinar AR42J cells (pancreatoma, ATCC CRL 1492) were cultured in Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO, USA) supplemented with 20% fetal bovine serum (GIBCO-BRL, Granad Island, NY, USA) and antibiotics (100 U/ml penicillin and 100 g/ml streptomycin) in a 10% CO_2 and 90% O_2. Induction of acute pancreatitis is achieved by stimulation of supramaximal concentration of cerulein (10^{-8} M).

2. Preparation of cytosolic and membrane fraction

The AR42J cells were trypsinized and washed with phosphate buffered saline (PBS) and then centrifuged at 500 rpm for 5min. The cells were resuspended with lysis buffer containing 10 mM Tris buffer pH 7.4, 50 mM NaCl, 1 mM EDTA, 50 μM leupeptin, 50 μM aprotitin, and 1 mM phenylmethylsulfonylfloride. And cells were lysed by drawing the cells through a 1 ml syringe with several rapid strokes. Nuclei and intact cells were sedimented down at 2,000 x g for 10 min. Membrane and cytosolic fraction were separated further by centrifugation at 100,000 x g for 1 hr. The protein content was determined by using a BCA protein assay kit[50] (Bio-rad laboratories, Hercules, CA, USA).

3. Transfection

Subconfluent AR42J cells were plated in 10-cm culture dishes. To observe signaling of cytokine expression, the cells were transfected for 24 hr with 10 μg of pcDNA, IκB mutant, RasN-17 or TAM67 expression plasmid constructs

using DOTAP (Boehringer Mannheim, Mannheim, Germany). On the other hand, to investigate signaling by VEGF-D, the cells were transfected for 24 hr with 500 μM of VEGF-D sense (5'-CAAAATGTA TGGAGAGTGGG-3') or 500 μM of antisense (5'-CCCACTCTCCATACATTTTG-3') oligodeoxynucleotide (GIBCO-BRL, Granad Island, NY, USA).

4. Reverse transcription-polymerase chain reaction

Total RNA is isolated from cells by guanidine thiocyanate extraction method[40]. cDNA was synthesized with 2 μg total RNA. Polymerase chain reaction (PCR) was carried out with 160 ng cDNA and cytokine (IL-1β, IL-6, TNF-α) primer. Total volume of PCR was adjusted to 20 μl with 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 0.1 mM dNTP, and 0.6U Tag polymerase. The internal control (GAPDH) is coamplified with a cytokine. Expression level of cytokine mRNA is normalized to that of housekeeping gene (GAPDH) in the same sample. The sequence for IL-1β primer is 5'-TCCTAGGAA ACAGCAATGGTCG-3'(forward primer) and 5'-TTCATCCCATACACACGGACAAC-3'(reverse primer). The sequence for IL-6 primer is 5'-CTGGTCTTCTGGAGTTCCGTT TC -3'(forward primer) and 5'-CATAGCACACTAGGTTTGCCGAG-3'(reverse primer). The sequence for TNF-α is 5'-CTCAAAGACAACCAACTGGTGGTAC-3'(forward primer) and 5'-ACAGAGCAATGACTCCAAAGTAGACC-3'(reverse primer). GAPDH sequence is 5'-ACCACAGTCCATGCCATCAC-3'(forward primer) and 5'-TCCACCACC CTGTTGCTGTA-3'(reverse primer). PCR amplification was performed employing the following conditions: denaturizing at 95°C for 30 sec, annealing at 60°C for 30 sec, and extension at 72°C for 30 sec for a total of 32 cycles. Five-micro liter samples

from each 25 μl PCR product were removed and analyzed by electrophoresis and UV transillumination on 1.5 % agarose gel involving 0.5 μg/ml ethidium bromide.

5. Enzyme-linked immunosorbent assay

The medium was immediately removed from cultures, and centrifuged at 15,000 x g to remove cell pellets and cellular debris. Levels of IL-6, IL-1β and TNF-α in medium were determined using enzyme-linked immunosorbent assay (ELISA) kit (Bio-source Inc., Canton, MA, USA) according to the manufacturer's instructions.

6. Western blotting analysis

Proteins in cell lysate, cytosolic fraction or membrane fraction were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE containing 10% polyacrylamide). After electrophoresis, proteins were transferred onto nitrocellulose membrane (Amersham, Arlington Heights, IL, USA) by semidry blotting system (Bio-rad laboratories, Hercules, CA, USA). After transfer of proteins onto nitrocellulose membrane, non-specific sites were blocked by a solution of Tris-buffered saline with Tween 20 (TBS-T: 50 mM Tris, 150 mM NaCl, 0.02 % (v/v) Tween-20, pH 7.4), containing 5 % (w/v) nonfat dry milk, for 2 hr at room temperature with gentle agitation. The nitrocellulose membrane was then incubated with specific antibody at a concentration of 0.2 μg/ml (1:1000) in TBS-T containing 5 % (w/v) nonfat-dry-milk for 12 hr at 4 °C. The nitrocellulose membrane was washed with TBS-T. Following the incubation of the nitrocellulose membrane with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG for 2 hr (1:2000 dilution in TBS-T containing 5 % (w/v) nonfat-dry-milk), the

nitrocellulose membrane was washed eight times for 10 min with TBS-T. Immunoreactive proteins were visualized by enhanced chemiluminescence (Amersham, Arlington Heights, IL, USA).

7. Electrophoretic mobility shift assay

Enzyme-linked immunosorbent assay (EMSA) was performed with nuclear extracts prepared from non-stimulated cells or cerulein-stimulated cells. Briefly, 30 μg of nuclear extract was incubated with ^{32}P-labeled probes in binding buffer (20 mM HEPES (pH 7.4), and 5 mM EDTA, 300 mM KCl, 25 % glycerol) for 40 min at room temperature. γ-^{32}P-labeled probes contained the following double-stranded oligonucleotides, which were generated by a kinase reaction with polynucleotide kinase and [γ-^{32}P] ATP (Amersham, Piscataway, NJ, USA) NF-κB (5'–AGTTGAGGGGACTTTCCCAGGC-3'), AP-1 (5'-CGCTTGATGACTCAGCCGGAA-3'). Reaction mixtures were analyzed on 6% polyacrylamide gels containing 0.25M TBE buffer (22.5 mM tris, 22.5 mM borate and 500 μM EDTA, pH 8.0). The gel was then dried for 1 hr at 80 °C and exposed to Kodak film (Eastman Kodak Co., Rochester, NY, USA) at −80 °C.

8. Proteomics

A. Isoelectric focusing and two-dimensional electophoresis separation

Three hundred μg of membrane fraction isolated from cells were adsorbed onto a 17 cm IPG strip (pH 5-8), and then electrophoresed on an isoelectric focusing (IEF) cells (Bio-Rad Laboratories, Hercules, CA, USA) for 70,000 Vh at 20 °C. Following IEF the IPG strips were subjected to equilibration for

15 min in equilibration buffer (375 mM Tris-HCl, pH 8.8, containing 6 M urea, 2 % w/v SDS, 20 % v/v glycerol, and 2 % w/v dithiothreitol). Strips were then re-equilibrated for 15 min in the same buffer containing 2.5 % w/v iodoacetamide in place of dithiothreitol. In all cases, molecular weight separation was achieved with 8 % polyacrylamide gel using Protean II xi cell gel SDS-PAGE system (Bio-Rad Laboratories, Hercules, CA, USA).

B. Coomassie brilliant blue-G 250 staining and image analysis

Proteins in gels were Coomasie brilliant blue (CBB) G-250 stained. After overnight fixation (50 % ethanol, 2 % w/v phosphoric acid), gels were washed three times for 20 min in double-distilled water and incubated for at least 48 hr in a solution containing 34 % methanol, 17 % ammonium sulfate, 3 % w/v phosphoric acid and CBB G-250 powder (0.1 %). The stained gels were digitalized using a GS 690 Imaging densitometer (Bio-Rad Laboratories, Hercules, CA, USA) at a resolution of 400 x 400 dpi. The digitalized images from both the CBB G-250 stained gels (from non-stimulated cells and cerulein-stimulated cells) were analyzed using the two-dimensional electrophoresis gel analysis program PDQuest (Bio-Rad Laboratories, Hercules, CA, USA). A comparison report of qualitative and quantitative differences of the samples for each set of data was then generated.

C. In-gel digestion

In-gel digestion of proteins from CBB G-250 stained gels was performed as follows. Spots were excised to 1-2 mm^2 slices using a blade, destained with freshly prepared 15 mM potassium ferricyanide/ 50 mM sodium thiosulfate, washed with 25 mM ammonium bicarbonate/ 50 % acetonitrile, and dried in a SpeedVac Plus SC100A vacuum concentrator (Savant, Holbook, NY, USA).

The dried gel pieces were rehydrated with 3-10 μl of 20 ng/ml trypsin solution, the solution volume being enough for the dried gel to be reswelled. Digestion was continued at 37 ^{o}C for 14-18 hr. Tryptic peptides were first extracted using 5 % trifluoroacetic acid (Bio-Rad Laboratories, Hercules, CA, USA) for 40 ^{o}C for 1 hr, then 2.5 % trifluoroacetic acid /50 % acetonitrile at 30 ^{o}C for 1 hr. The extracted solutions were mixed in an eppendorf tube, and dried in a vacuum concentrator.

D. Proteomic analysis

The peptide mixture was solubilized with 0.5 % trifluoroacetic acid for MS analysis. MS was performed on a Micromass M@LDI-TOF (Manchester, UK) with saturated α-cyano-4-hydroxy-trans-cinnamic acid solution in 0.1% trifluoroacetic acid / 50% acetonitrile as matrix. Mass spectra were externally calibrated with autodigest peaks of trypsin (MH+: 906.505 Da, 1020.504 Da, 1153.574 Da, 2163.057 Da, and 2273.160 Da). The peptide mass maps produced by MALDI-TOF MS were searched against the published databases by means of the MS-Fit module in Protein Prospector and Mascot.

9. Immunoprecipitation

It is investigated whether VEGF-D is associated with angiotensin II type 2 receptor using immunoprecipitation (IP) method. AR42J cells were stimulated with cerulein for 15, 30 and 60 min. The cells were washed with PBS and scraped. The cells were centrifugated at 1200 x g for 10 min and the supernatant was removed. The cells were lysated with 1 ml IP buffer containing 100 mM NaCl, 20 mM Tris buffer (pH 7.4), 1 mM EDTA, 1 mM EGTA, 0.1 % SDS, 1 % Nonidet P-40, 0.5 % deoxycholate, 50 μM leupeptin, 50 μM aprotitin, and 1 mM phenylmethylsulfonylfloride and incubated for 30

min on ice. The samples were centrifugated at 7500 x g for 30 min. Supernatant was transfered to new tube and its protein concentration was determined. 1 mg of protein was added to new tube and then incubated with anti- angiotensin II type 2 receptor antibody and protein A/G Plus-agarose (Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4 ^{o}C. After centrifugation, the pellet was washed with IP buffer and was used for western blotting analysis for VEGF-D.

10. Immunocytochemistry

Immunocytochemistry against cell type–specific markers was performed on AR42J cell cultures. AR42 J cells were cultured at cerulein for 1 hr, fixed in cold methanol for 10 min and washed three times with PBS. The cells were then incubated with polyclonal primary antibodies. Primary antibodies used were rat anti–rabbit VEGF-D (4 μg/ml; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and rat anti–goat angiotensin II type 2 receptor (4 μg/ml; Santa Cruz Biotechnology, Santa Cruz, CA, USA). After incubation of blocking solution (containing 2 % goat serum, 1 % BSA and 0.1 % gelatin in PBS) for 1 hr, cells were incubated for 1 hr at room temperature with secondary antibody solution containing FITC-conjugated secondary antibody (final concentration; 2 μg/ml in blocking solution) and rhodamine-conjugated secondary antibody (final concentration; 3 μg/ml in blocking solution) purchased from Jackson Immuno-Research Laboratories (USA). Samples were again washed in PBS and counterstained with mounting medium and cover-slipped. The cells incubated with FITC- and rhodamine-conjugated secondary antibodies were examined with laser scanning confocal microscope (Leica TCS-NT, Heidelberg, Germany) and photographed.

Optical filters used for exitation were 450-490 nm for FITC (green) and 510-

560 nm for rhodamine (red).

11. Immnoprecipitation for the protein on cell surface

To immunoprecipitate the populations of VEGF-D on the cell surface, cells were cultured with cerulein for 30 min, 1 hr, or 2 hr. Cultures were washed with cold PBS, and then incubated with an anti-VEGF-D antibody (2 μg/ml in 2 % bovine serum albumin in PBS) or normal goat IgG (2 μg/ml in 2 % bovine serum albumin in PBS) for 1 hr on ice with gentle shaking. The unbound antibody was removed with cold PBS and cells were lysed by adding extraction buffer containing 150 mM NaCl, 10 mM Tris-HCl buffer (pH 7.4), 1 mM EDTA, 1 mM EGTA, 0.5 mM phenylmethylsufonylfloride, 1 % Nonidet P-40, and 0.5 % deoxycholate. The insoluble material was separated by centrifugation (13,000 x g for 30 min at 4 °C). The clarified supernatant was incubated with 50 μl of protein A/G Plus-agarose per a sample for overnight at 4 ^{o}C. The beads were washed with extraction buffer time, and resuspended in 2x SDS sample buffer[46]. Levels of VEGF-D were determined by western blotting analysis using anti-VEGF-D antibody.

12. Statistical analysis

Each experiment was performed at least three times. Results were expressed as means ± standard error of four separate experiments. Analysis of variance (ANOVA) followed by Newman-Keul's test was used for statistical analysis[47]. $P<0.05$ was considered statistically significant.

III. RESULTS

1. Cerulein induces cytokine expression and activates NF-κB and AP-1 in AR42J cells

Cerulein increased the mRNA and protein levels of IL-6, IL-1β and TNF-α in AR42J cells over 0~4 hr of treatment (Fig.1A and B). The EMSA data showed that NF-κB and AP-1 were activated by cerulein within 30 min (Fig. 1C).

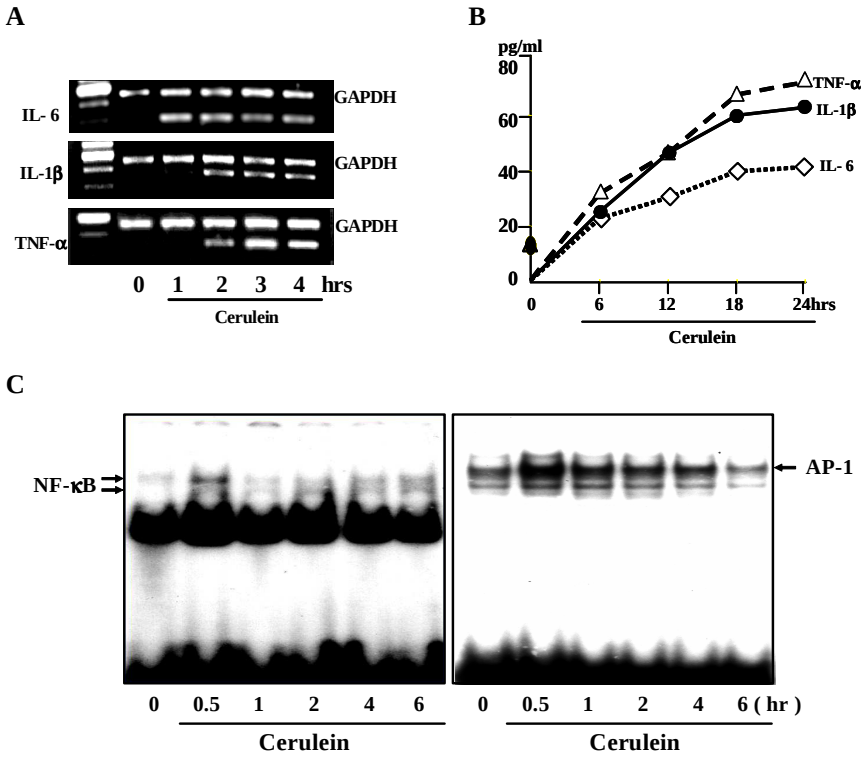
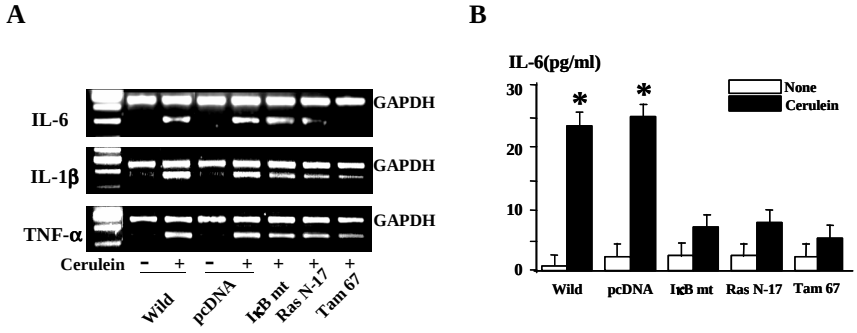


Fig.1**. Expression of cytokines and activation of NF-κB and AP-1 in cerulein-stimulated AR42J cells.** Cells were cultured in the absence or presence of cerulein

for the indicated times. (A) mRNA levels of cytokines were measured by RT-PCR. mRNA expression levels were normalized by internal control (GAPDH) in the same sample. (B) Levels of cytokines in medium were determined by ELISA. (C) DNA-binding activities of NF-κB and AP-1 in nuclear extracts were determined by EMSA analysis

2. Cytokine expression is mediated by Ras, AP-1, and NF-κB in cerulein-stimulated AR42J cells

Transfection with Ras N-17, TAM67 or IκB mutant gene significantly suppressed cerulein-induced IL-6 expression and, to a lesser extent, IL-1β and TNF-α expression (Fig. 2A-D). Cerulein-induced DNA binding activities of NF-κB and AP-1 were significantly reduced by transfection with Ras N-17 and TAM67 (Fig. 2E). Transfection with IκB mutant gene attenuated NF-κB activation but not that of AP-1. These data shows that DNA binding activity of NF-κB is regulated by AP-1 and Ras in cerulein-stimulated AR-42J cells and suggests possible crosstalk between AP-1, NF-κB, and MAP kinase in cytokine expression in AR42J cells.



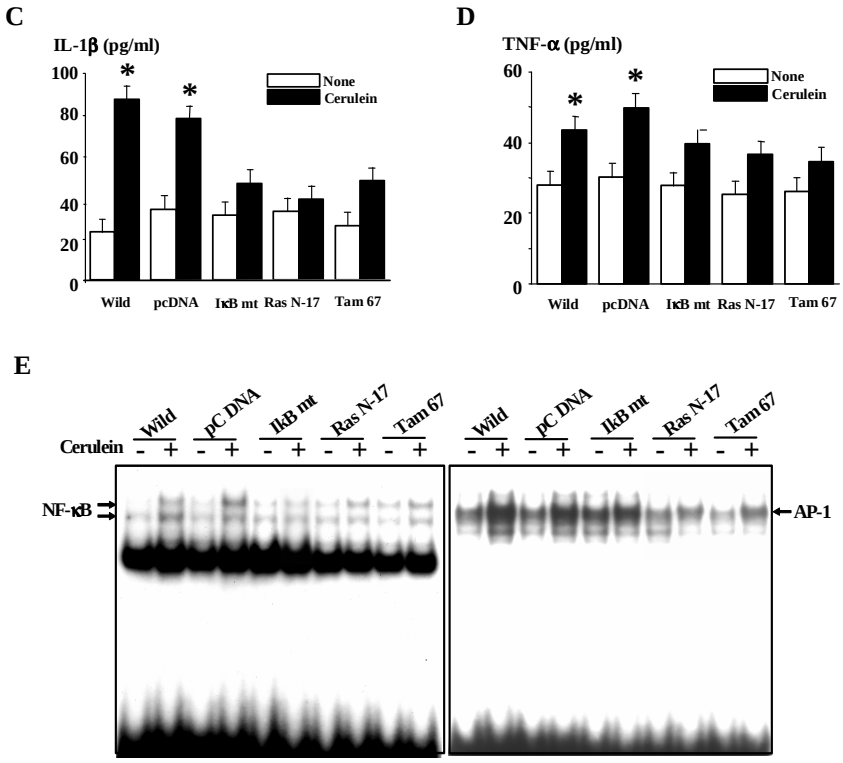


Fig. 2. **Expression of cytokines and activation of NF-κB and AP-1 in cerulein-stimulated AR42J cells transfected with IκB mutant, Ras N-17 or TAM 67.** Before stimulation of cerulein, cells were transfected with IκB mutant, Ras N-17, or TAM 67 for 24 hr. (A) For the analysis of cytokine mRNA expression, cells were stimulated with cerulein for 3 hr. Cytokine mRNA expression levels were normalized by internal control GAPDH in the same sample. (B) Cytokine production was measured after 24 hr treatment with cerulein and compared with non-treated group. *P<0.05 vs wild none or pcDNA (without cerulein) (C) After 30 min stimulation with cerulein, activation of NF-κB and AP-1 was determined by EMSA analysis.

3. Cytokine expression is mediated by MAP kinases in cerulein-stimulated AR42J cells

To clarify the role of activated p38 and ERK1/2 in cerulein-induced cytokine expression, cells were treated with SB203580, U0126 or PD98059 prior to cerulein treatment. Cerulein-induced expression of cytokine mRNA was suppressed by treatment of U0126 or PD98059 (Fig. 3A). Cerulein-induced DNA binding activities of NF-κB and AP-1 were also reduced by U0126 and PD98059 (Fig. 3B). This result suggests that expression of cytokines and activations of NF-κB and AP-1 are mediated by ERK1/2 in cerulein-stimulated AR42J cells.

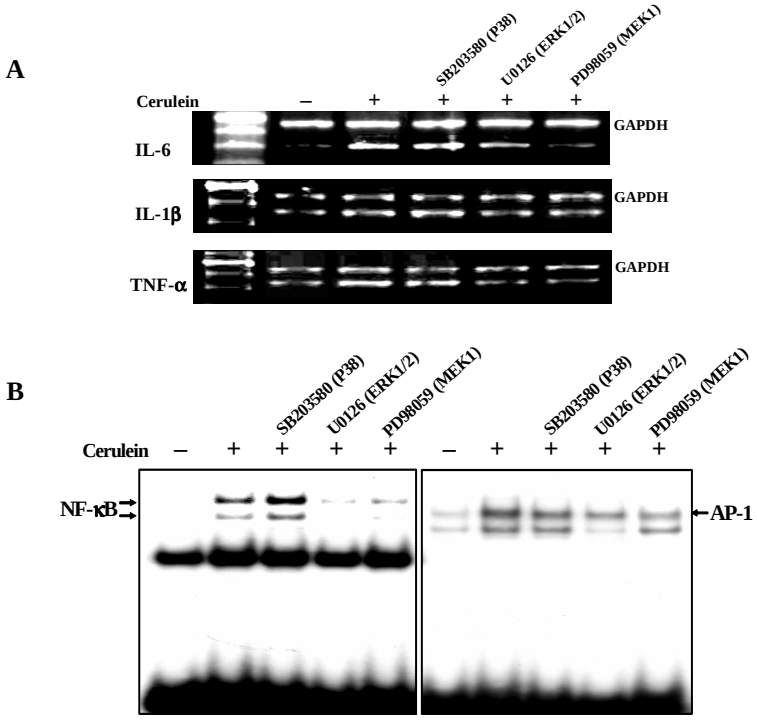


Fig. 3. **Effect of ERK or p38 antagonist on expression of cytokines and activation of NF-kB and AP-1 in cerulein-stimulated AR42J cells.** SB203580, U0126 and

PD98059 are inhibitors for p38, ERK1/2, and MEK1, respectively. Before stimulation of cerulein, cells were treated with antagonist for 10 min. (A) For cytokine mRNA expression, cells were incubated in absence or presence of cerulein for 3 hr. Cytokine mRNA expression levels were normalized by internal control GAPDH in the same sample. (B) After 30 min stimulation with cerulein, activation of NF-κB and AP-1 was determined by EMSA analysis.

4. Cerulein induces differential expression of membrane proteins of AR42J cells

Membrane proteins play important roles in various cellular processes including signal transduction. Thus, the relationship between differentially expressed membrane proteins and induction of acute pancreatitis may be the early important pathologic event in inflammatory cytokine expression. Membrane proteins were extracted from non-stimulated cells or cerulein-stimulated cells. Isoelectric focusing analysis and two-dimensional electophoresis separation were carried out to determine proteins differentially expressed by cerulein. Differentially expressed membrane proteins were identified by peptide mass fingerprinting (Fig. 4). Mascot search using the peptide mass fingerprinting data indicated that the differentially expressed proteins are protein disulfide isomerase precursor, actin beta, MASP-2 protein, Hsp 60, phosphatidylinositol transfer protein, actin gamma, isocitrate dehydrogenase 3 (NAD+) alpha, acidic ribosomal protein PO, tumor-associated Ca2+ signal transducer 1, seven in absentia 1A, ER protein 29, ATP synthase subunit d (Table 1).

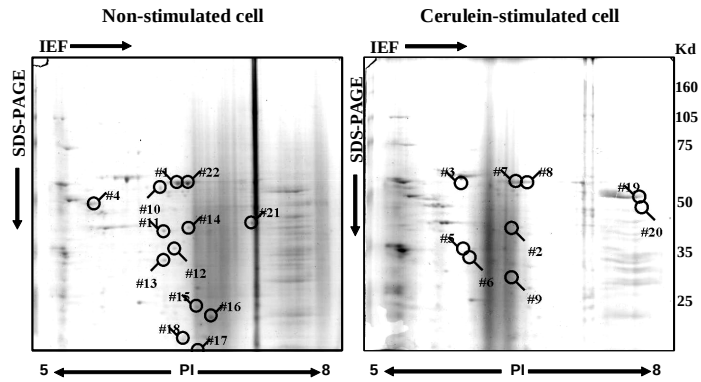


Fig. 4. **Proteomic analysis of membrane proteins in non-stimulated cells or cerulein-stimulated AR42J cells.** Two-dimensional gel map was derived from non-stimulated cells or cerulein-stimulated cells for 1 hr. Three hundred μg of membrane protein isolated from cells was applied to pH 5-8 linear IPG strips (17 cm), and with 8 % linear vertical SDS-PAGE as the second dimension. The gel was visualized by CBB-G staining. Several proteins marked above are those that were differentially expressed between non-stimulated cells and cerulein-stimulated cells.

Table 1. **List of membrane proteins changed by cerulein in AR 42J cells.**

Spot No.	Protein name	Protein database accession No.	Predicted M r	Predicted pI	% Coverage	Change by cerulein
R01	Protein disulfide isomerase A3 precursor	1352384	56624	5.9	56.0	Decreased
R02	Actin beta	71620	41751	5.3	50.0	Increased
R03	Protein disulfide isomerase A3 precursor	129731	56952	4.8	22.0	Increased
R04	Protein disulfide isomerase A3 precursor	2501206 M	47221	4.9	32.0	Decreased
R05	VEGF-D	15430279	37107	5.9	32.0	Increased
R06	MASP-2 protein	6689087	36951	6.0	37.0	Increased
R07	Heat shock protein 60	11560024 M	60966	5.9	42.0	Increased
R08	Heat shock protein 60	11560024 M	60966	5.9	46.0	Increased
R09	Phosphatidylinositol transfer protein	16758568 M	31450	6.4	25.0	Increased
R11	Actin, gamma	4501887 M	41793	5.3	46.0	Decreased
R12	Isocitrate dehydrogenase 3 (NAD+) alpha	16758446 M	39614	6.5	44.0	Decreased
R13	Acidic ribosomal protein PO	11693176 M	34216	5.9	64.0	Decreased
R14	Tumor-associated Ca^{2+} signal Transducer 1	25742698 M	35208	6.0	35.0	Decreased
R15	Seven in absentia 1A	18266718 M	32017	6.7	30.0	Decreased
R16	Endoplasmic retuclum protein 29	16758848 M	28575	6.2	51.0	Decreased
R17	ATP synthase subunit d	9506411 M	18764	6.2	58.0	Decreased
R22	Protein disulfide isomerase A3 precursor	1352384	56624	5.9	46.0	Decreased

5. Cytokine expression is mediated by angiotensin II type 2 receptor but not by VEGF receptor in cerulein-stimulated AR42J cells

To investigate whether activation of angiotensin II type 2 receptor and/or VEGF receptor are involved in cerulein-induced inflammation, cells were stimulated with an angiotensin II type 2 receptor or a VEGF receptor antagonist prior to cerulein-stimulation. Cerulein-induced cytokine expression was suppressed by an angiotensin II type 2 receptor antagonist but not by VEGF receptor antagonist (Fig. 5). This result suggests that cerulein-induced cytokine expression is mediated by angiotensin II type 2 receptor but not by VEGF receptor. It is possible that VEGF-D may mediate cerulein-induced inflammation through interacting with angiotensin II type 2 receptor because VEFG-D was known to bind with angiotensin II type 2 receptor as well as VEGF receptor.

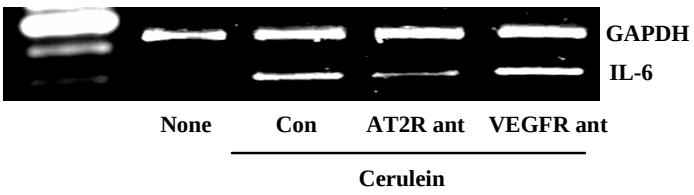


Fig. 5. **Effects of angiotensin II type 2 or VEGF receptor antagonist on IL-6 mRNA expression in cerulein-stimulated AR42J cells.** For IL-6 mRNA expression, cells were treated with antagonists for 15 min prior to 3hr stimulation with cerulein. Angiotensin II type 2 receptor antagonist (AT2R ant) is PD123319, and VEGF receptor antagonist (VEGFR ant) is total subtype of VEGF receptor's antagonist. Cytokine mRNA expression levels were normalized by internal control GAPDH in the same sample.

6. Cerulein induces VEGF-D expression

To determine whether cerulein induces VEGF-D expression, the level of VEGF-D in cerluein-stimulated cells was determined using western blotting analysis. Level of VEGF-D was significantly increased after 30 min of cerulein stimulation whereas that of angiotensin II type 2 receptor was unchanged.

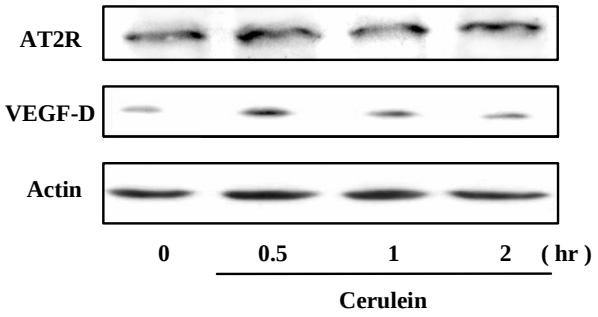


Fig. 6. **VEGF-D expression in cerulein-stimulated AR42J cells.** Cells were stimulated with cerulein at the indicated times. Levels of angiotensin II type 2 receptor and VEGF-D in whole cell lysates were determined by western blotting analysis using specific antibodies.

7. VEGF-D interacts directly with angiotensin II type 2 receptor in cerulein-stimulated AR42J cells

To investigate whether the VEGF-D is associated with activation of angiotensin II type 2 receptor in cerulein-stimulated AR42J cells, it was examined whether VEGF-D directly binds to angiotensin II type 2 receptor using immunoprecipitation method. VEGF-D was detected in anti-angiotensin II type 2 receptor immunoprecipitates in cerulein-stimulated

AR42J cells. This result indicates that angiotensin II type 2 receptor interacts with VEGF-D in cerulein-stimulated AR42J cells.

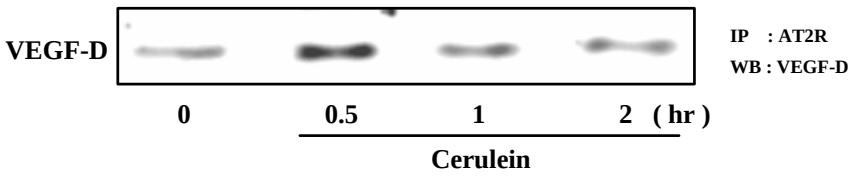


Fig. 7. **Interaction of VEGF-D with angiotensin II type 2 receptor in cerulein-stimulated AR42J cells.** The whole lysate was prepared from the cells at the indicated times and was immunoprecipitated for angiotensin II type 2 receptor using anti- angiotensin II type 2 receptor antibody. Level of VEFG-D in immunoprecipitates was determined by western blot using anti-VEGF-D antibody.

8. Membrane translocation of VEGF-D is increased in cerulein-stimulated AR42J cells

To demonstrate the membrane translocation of VEGF-D by cerulein, level of VEGF-D in cytosolic and membrane fractions from non-stimulated or cerulein-stimulated cells were determined by western blotting. Level of VEGF-D in membrane was increased between 30 min and 60 min while that of VEGF-D in cytoplasm was decreased (Fig. 8A). This was confirmed with immunocytochemistry assay (Fig. 8B). This indicates that membrane translocation of VEGF-D is increased in cerulein-stimulated AR42J cells.

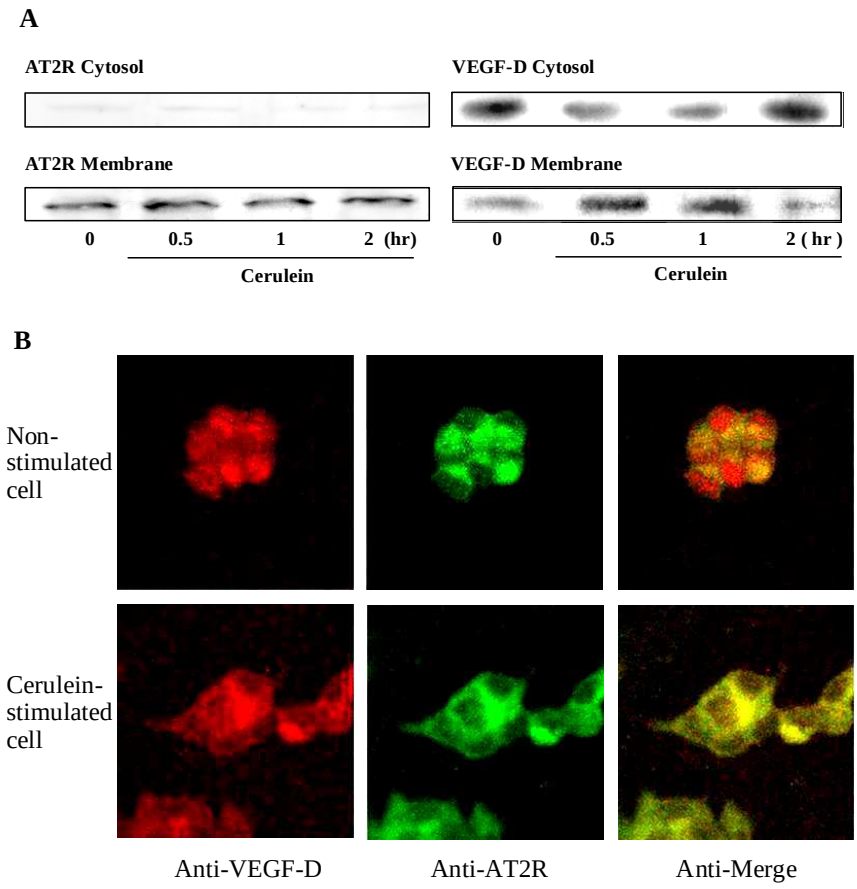


Fig. 8. **Translocalization of VEGF-D in cerulein-stimulated AR42J cells.** (A) AR42J cells were cultured in the absence or presence of cerulein. Cells were collected at indicated times. After isolation of membrane and cytosolic fractions from cells, levels of VEGF-D and angiotensin II type 2 receptor in membrane and cytosolic fractions were determined by western blotting analysis. (B) The cells were cultured in the absence or presence of cerulein for 1 hr. Cells were incubated with anti-angiotensin II type 2 receptor or VEGF-D antibody, washed with PBS and then incubated with FITC-conjugated (green) or rodamine-conjugated (red) secondary antibody.

9. VEGF-D is translocated to cell surface in cerulein-stimulated AR42J cells

It was investigated whether VEGF-D is translocated to cell surface by cerulein-stimulation. VEGF-D in cell surface was immunoprecipitated using anti-VEGF-D antibody and then detected by western blotting analysis. VEGF-D in cell surface was increased by cerulein (Fig. 9). This indicates that VEGF-D is translocated to cell surface by cerulein.

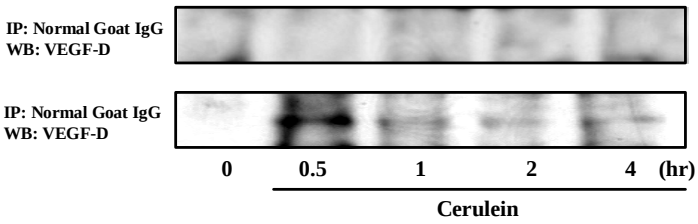


Fig. 9. **Immunoprecipitation of VEGF-D in cell surface.** AR42J cells were cultured in the absence or presence of cerulein for various time periods. The intact cells were incubated with normal goat IgG or an anti-VEGF-D antibody (2 μg/ml in 2% bovine serum albumin in PBS) for 60 min on ice with gentle shaking. VEGF-D in cell surface was immunoprecipitated with an anti-VEGF-D antibody as described in Materials and Methods. Immunoprecipitates was prepared in parallel with non-immune IgG. The immunoprecipitate were subjected to western blotting analysis using anti-VEGF-D antibody.

10. DNA binding activation, cytokine expression, and MAP kinase phosphorylations are mediated by VEGF-D in cerulein-stimulated AR42J cells

To determine the role of VEGF-D in cerulein-induced inflammation,

antisense-oligodeoxynucleotide for VEGF-D was transfected into cells. Antisense-oligodeoxynucleotide for VEGF-D suppressed activation of NF-κB and AP-1 (Fig. 10) as well as mRNA expression of IL-6 (Fig. 11) in cerulein-stimulated AR42J cells. Additionally, antisense-oligodeoxynucleotide for VEGF-D suppresses MAP kinases phosphorylation in cerulein-stimulated cells (Fig. 12).

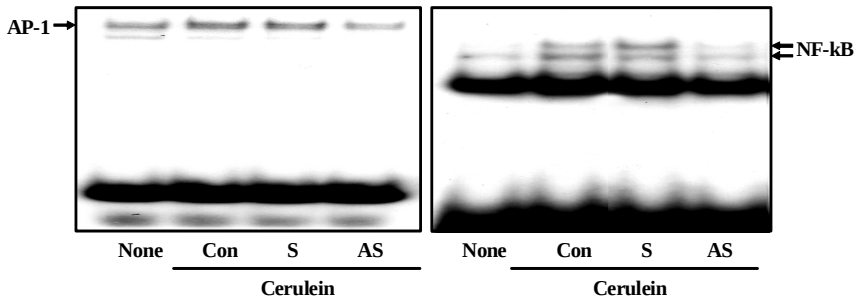


Fig. 10. **Activation of NF-κB and AP-1 in cerulein-stimulated AR42J cells transfected with antisense-oligodeoxynucleotide for VEGF-D.** The cells were transfected with 500 μM sense(S)- or antisense(AS)-oligodeoxynucleotide for VEGF-D and then cultured in the absence or presence of cerulein for 30 min. DNA binding activities for f NF-κB and AP-1 were determined by EMSA.

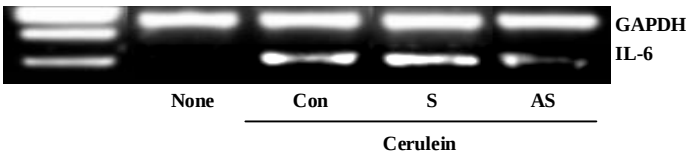


Fig. 11. **IL-6 mRNA expression in cerulein-stimulated AR42J cells transfected with antisense-oligodeoxynucleotide for VEGF-D.** The cells were transfected with 500 μM sense(S)- or antisense(AS)-oligodeoxynucleotide for VEGF-D using DOTAP

reagent and then cultured in the absence or presence of cerulein for 3 hrs. Level of IL-6 mRNA was determined by RT-PCR. The internal control (GAPDH) was coamplified. IL-6 mRNA expression level were normalized to that of housekeeping gene GAPDH in the same sample.

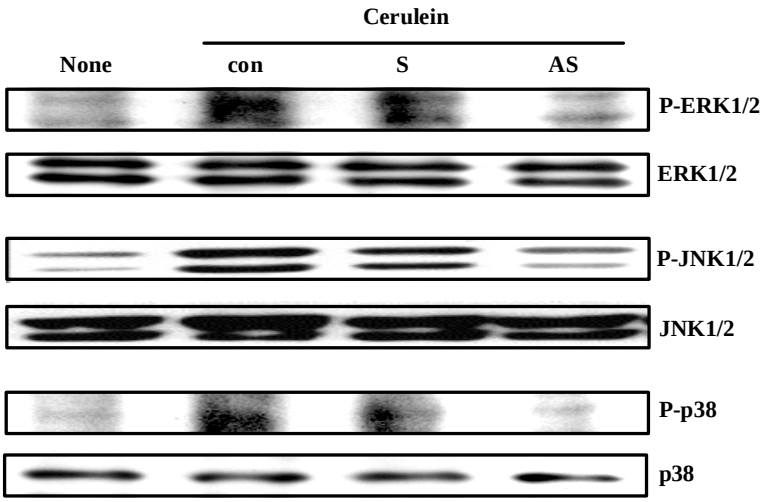


Fig. 12. **MAP kinases phosphorylation in cerulein-stimulated AR42J cells transfected with antisense-oligodeoxynucleotide for VEGF-D.** The cells were transfected with 500 μM sense(S)- or antisense(AS)-oligodeoxynucleotide for VEGF-D and then cultured in the absence or presence of cerulein for 15 min. Levels of phosphorylated or nonphosphorylated forms of ERK1/2, JNK1/2 and p38 were determined by western blotting analysis.

IV. DISCUSSION

AR42J is a pancreatic acinar cell line obtained from primary culture of isolated rat pancreatic acinar cells after induction with azaserine. Despite a few disadvantages like short survival period or high baseline cytokine expression and p38 activation, AR42J is widely used to study pancreatic exocrine system because it is the only cell line which maintains distinctive characteristics of pancreatic acinar cells such as synthesis/secretion of digestive enzymes and expression profile of major receptors.

Cytokines which are produced in pancreatic acinar cells induce inflammatory cell infiltration into pancreas, producing an inflammatory response. The cellular mechanism regulating these inflammatory molecules involves activation of transcription factors such as NF-κB and AP-1[18, 19]. Our group previously reported that expression of inflammatory cytokines was mediated by cerulein-activated NF-κB and AP-1 in pancreatic acinar cells[51].

In present study, expression of IL-6, TNF-α, and IL-1β was suppressed by dominant negative mutant of NF-κB, AP-1 and Ras in cerulein-stimulated AR42J cells. This demonstrates that the expression of inflammatory cytokines is mediated by NF-κB, AP-1 and Ras in cerulein-stimulated AR42J cells. The activation of NF-κB was inhibited by dominant negative mutant of AP-1 whereas that of AP-1 was not inhibited by dominant negative mutant of NF-κB. This suggests that activation of NF-κB may be mediated by AP-1 and Ras in cerulein-stimulated AR-42J cells. Additionally, ERK1/2 inhibitor suppressed cerulein-induced activation of both NF-κB and AP-1. This result suggests that activation of NF-κB and AP-1 should be mediated by ERK1/2 in cerulein-stimulated pancreatic acinar cell. Several investigators reported that

activation of AP-1 is mediated by ERK1/2. It was also known that the active ERK1/2 directly phosphorylate and degrade IκB, and then activate NF-κB in rat vascular smooth muscle cells. These results suggest that cerulein induces the activation of not only NF-κB, but also AP-1 through ERK signaling pathways.

It is well known that cerulein induces inflammatory signaling in pancreatic acinar cells through its interaction with CCK-A or CCK-B receptor. However, it was recently reported that the pancreas possesses a local renin-angiotensin system, which performs various activities in the regulation of exocrine and endocrine functions of the pancreas[27]. In the exocrine pancreas, both angiotensin II type 1 receptor and angiotensin II type 2 receptor are localized in the pancreatic ducts and acinar cells. Indeed, renin-angiotensin system components are present in AR42J cell line as well as isolated acinar cells [26]. Available data suggest that a local RAS in the exocrine pancreas should be involved in the regulation of pancreatic microcirculation, acinar enzyme secretion, and ductal anion secretion via mediation of angiotensin II type 2 receptor[26, 27]. Interestingly, pancreatic inflammatory conditions such as pancreatitis, hypoxia, and fibrosis can upregulate the renin-angiotensin system components in the pancreas, leading to further inflammation and tissue injury[13, 26]. In 1991, it was published that key components for inflammation comprise an intrinsic renin-angiotensin system within the canine pancreas[32]. Subsequent studies by other investigators reported comparable findings in the rat, mouse and human pancreas. Indeed, it was shown that the AR42J cell line expresses all components of an renin-angiotensin system including renin, angiotensinogen, angiotensin converting enzyme (ACE), angiotensin II type 1 receptor and angiotensin II type 2 receptor. Thus, these cells may be of particular value to study the interplay of the angiotensin II type 1 receptor and

angiotensin II type 2 receptor in regulation of cell growth and potentially exocrine function[26].

In present study, cerulein increased membrane translocation of VEGF-D and interaction of VEGF-D with angiotensin II type 2 receptor in AR42J cells. This result indicates that VEGF-D, in conjunction with angiotensin II type 2 receptor, may play an important role in cerulein-induced pancreatitis[52]. It was previously reported in our laboratory that inflammatory cytokine expression is mediated by NF-κB in cerulein-stimulated pancreatic acinar AR42J cells[51]. Present study demonstrates that cerulein-induced cytokine expression is mediated by Ras- AP-1- NF-κB signaling in cerulein-stimulated AR42J cells. Additionally, this study provides evidence that both VEGF-D and angiotensin II type 2 receptor play significant roles in inflammatory signaling of AR42J cells. Levels of VEGF-D in membrane, and its interaction with angiotensin II type 2 receptor were increased by cerulein. Inhibition of membrane translocation of VEGF-D by its antisense-oligodeoxynucleotide suppressed activations of NF-κB and AP-1, MAP kinase phosphorylation and cytokine expression in cerulein-stimulated AR42J cells. Furthermore, cerulein-induced cytokine expression was inhibited by angiotensin II type 2 receptor antagonist but not by VEGF-D receptor antagonist. These results suggest that angiotensin II type 2 receptor and VEGF-D are involved in cerulein-induced cytokine expression in pancreatic acinar AR42J cells. Therefore, this study suggests that VEGF-D induces novel cerulein-induced pancreatits pathway via interaction with angiotensin II type 2 receptor. VEGF-D-activated angiotensin II type 2 receptor may have a novel role in the development of pancreatitis.

V. CONCLUSION

Cerulein pancreatitis is similar to human acute pancreatitis with dysregulation of the digestive enzyme production and cytoplasmic vacuolization, the death of acinar cells, edema formation, and infiltration of inflammatory cells into the pancreas. In this study, we identified several differentially expressed proteins in cerulein-stimulated AR42J cells, which are related to cellular stress, cytoskeletal function, and cell signaling. Among these proteins, VEGF-D is involved in regulation of inflammatory signaling in pancreatic AR42J cells. The present study demonstrated that VEGF-D binds angiotensin II type 2 receptor and controls cytokine expression in pancreatic AR42J cells. Moreover we showed that VEGF-D mediates cerulein-induced inflammatory signaling such as NF-κB and AP-1 through interaction with angiotensin II type 2 receptor. In conclusion, this study suggests that VEGF-D is involved in new cerulein-induced pancreatits pathway via the interaction with angiotensin II type 2 receptor in pancreatic acinar cells. VEGF-D-activated angiotensin II type 2 receptor may have a novel role in the development of pancreatitis.

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Abstract (in Korean)

췌장 선세포에서의 급성 췌장염 유발에 대한 Angiotensin II type 2 수용체 및 VEGF-D 에 대한 역할 규명

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이 장 원

급성 췌장염은 최근의 많은 기초적, 임상적 연구에도 불구하고 아직까지도 높은 사망률을 보이며 정확한 병리기전은 잘 알려져 있지 않은 실정이다. Cholecystokinin 유도체인 cerulein은 빠른 시간에 인체 급성 췌장염에서 볼 수 있는 췌장효소의 과잉 분비를 유도하여, 급성 췌장염 징후 현상들(췌장의 부종, 선세포의 vacuolization, 혈청 amylase와 lipase의 증가, 출혈 및 조직의 괴사)을 나타내므로, cerulein 유도 급성 췌장염 모델은 급성췌장염의 모델로써 널리 사용되고 있다.

본 연구에서는 cerulein의 처치에 의하여 급성 췌장염을 유발시킨 췌장선세포주인 AR42J 세포를 이용하여 췌장염 유발에 의한 angiotensin II type 2 수용체 활성화의 기전에 대하여 VEGF-D의 상호관련성을 규명하여 급성 췌장염 유발에 대한 angiotensin

II type 2 수용체 활성에 대한 새로운 기전을 규명하고자 하였다. 췌장염의 발생 기전에 대한 예비실험으로서 cerulein을 처치한 AR42J 세포주에서 막단백질을 분리한 후 2D gel 전기영동법을 사용하여 이들 단백질들을 분석하여 본 결과 여러 세포막의 단백질의 발현량의 변화를 관찰하였는데, 이 중 VEGF-D 단백질이 증가함을 보였다. 다른 세포의 염증관련 신호전달체계에서 혈관내피세포성장인자로 알려진 이 단백질은 angiogenesis에서의 성장인자 및 ERK1/2 활성화에 영향을 주는 것으로 알려져 있다. 최근 연구 결과, 또 다른 rennin-angiotensin 계의 요소인 angiotensin II type 2 수용체가 췌장에서의 존재가 확인되어 이 단백질도 cerulein 유도 췌장염 유발의 신호전달체계에 관여하고 있음을 시사하고 있다. 또한 췌장염은 angiotensin II type 2 수용체의 활성화에 의한 MAP kinase인 ERK1/2 활성에 의하여 유발된다고 알려졌다. 게다가 VEGF-D가 이 angiotensin II type 2 수용체와 단백질 결합을 통하여 염증을 유발한다는 보고는 있지만, 염증 초기 단계의 췌장염에서의 조절기전에 대해서 명확한 결과를 제시하지 못하고 있어, 그 체계적인 연구가 아직까지 미미한 실정이다.

본 실험 결과, cytokine의 발현은 ERK1/2 또는 MEK1의 길항제와 마찬가지로 IκB 변종, RasN-17 또는 TAM67 같은 dominant negative 유전자에 의하여 억제됨을 보아, cerulein 유도 염증 유발에는 NF-κB, AP-1와 ERK1/2가 관여함을 알 수 있다. 또한, cerulein에 의한 NF-κB and AP-1에 대한 DNA 결합 활성도는 ERK1/2의 길항제에 의하여 억제됨을 보아, NF-κB and AP-1에 대한

DNA 결합 활성도는 ERK에 의한 신호전달체계에 의해 매개됨을 시사한다.

Cerulein에 의하여, 세포막에서의 VEGF-D의 양적 증가와, VEGF-D와 angiotensin II type 2 수용체의 상호간 단백결합이 이루짐을 관찰할 수 있었다. VEGF-D의 antisense-oligodeoxynucleotide을 세포내로 transfection 시키어 VEGF-D의 세포막 이동을 억제시키면 NF-κB and AP-1에 대한 DNA 결합 활성도와, MAP kinases의 인산화, IL-6 발현이 억제되는 것으로 보아, VEGF-D의 cerulein에 의한 세포막 이동이 중요한 역할을 할 것으로 보인다. 또한, cerulein으로 유도된 cytokine 발현은 VEGF-D 수용체 억제제가 아닌 angiotensin II type 2 수용체의 억제제에 의하여 감소되었음을 보였다. 결론적으로 지금까지의 췌장염 병인 연구의 결과에 의하면 cerulein의 자극으로 cholecystokinin 수용체의 활성화를 통하여 친염증성 cytokine의 발현하는 신호전달체계 외에, cerulein에 의한 VEGF-D와 세포막의 angiotensin II type 2 수용체의 결합을 통하여 친염증성 cytokine의 발현으로 이루어지는 새로운 신호전달체계가 존재한다고 생각된다.

핵심되는 말: VEGF-D, Angiotensin II type 2 수용체, cerulein, 급성췌장염