Protease-activated Receptor 2 is Associated with Activation of Human Macrophage Cell Line THP-1

Chon-Sik Kang[1,3], Jin Tae[1], Young-Mi Lee[1], Byeong-Soo Kim[2], Woo-Sung Moon[4] and Dae-Ki Kim[3]

[1]Department of Oriental Pharmacy, College of Pharmacy, Wonkwang University, Iksan, [2]Department of Companion and Laboratory Animal Science, Kongju National University, Gongju, Departments of [3]Immunology and [4]Pathology, Chonbuk National University Medical School, Jeonju, Korea

ABSTRACT

Background: Protease-activated receptor 2 (PAR2) belongs to a family of G protein-coupled receptors activated by proteolytic cleavage. Trypsin-like serine proteases interact with PAR2 expressed by a variety of tissues and immune cells. The aim of our study was to investigate whether PAR2 stimulation can lead to the activation of human macrophages. **Methods:** PAR2-mediated proliferation of human macrophage cell line THP-1 was measured with MTT assay. We also examined the extracellular regulated kinase (ERK) phosphorylation and cytokine production induced by trypsin and PAR2-agonist using western blot and enzyme-linked immunosorbent assay (ELISA), respectively. **Results:** Treatment of trypsin or PAR2-activating peptide increased cell proliferation in a dose-dependent manner, and induced the activation of ERK1/2 in THP-1 cells. In addition, trypsin-induced cell proliferation was inhibited by pretreatment of an ERK inhibitor (PD98059) or trypsin inhibitor (SBTI). Moreover, PAR2 activation by trypsin increased the secretion of TNF-α in THP-1 cells. **Conclusion:** There results suggest that PAR2 activation by trypsin-like serine proteases can induce cell proliferation through the activation of ERK in human macrophage and that PAR2 may play a crucial role in the cell proliferation and cytokine secretion induced by trypsin-like serine proteases. **(Immune Network 2005;5(4):193-198)**

Key Words: Protease-activated receptor 2, macrophage, trypsin, cell proliferation, extracellular regulated kinase

Introduction

Macrophages are widely distributed in all tissues and derived from precursors in bone marrow to constitute the mononuclear phagocyte system for host defense against infectious microorganisms. The activation of macrophages can secret a variety of mediators including nitric oxide (NO), proinflammatory cytokines, and chemokines, which are critical factors in host defense and inflammation (1).

Protease-activated receptors (PARs) were recently described as new subfamily of G protein-coupled receptors with seven transmembrane domains that are cleaved and activated by various serine proteases (2). It has been reported that serine proteases induce the cleavage of these receptors to regulate important biological effects such as inflammation, immune response, host defense, chemotaxis, cytokine and growth factor release, vascular function, tissue repair, and apoptosis (3). So far, the PAR family comprises 4 members, PAR1, PAR3, and PAR4 activated by thrombin (4), and PAR2 activated by trypsin and mast cell tryptase (5).

It is well known that the mast cell tryptase and extrapancreatic trypsin are released at sites of inflammation and are involved in inflammation response via activation of PAR2 (6-8). PAR2 is expressed in a variety of organ tissues including gastrointestinal tract and lung (3). We previously have shown that PAR-2 was highly expressed in the tissues of patient with ulcerative colitis (9). The inflammatory mediators, such as tumor necrosis factor (TNF)-α interleukin (IL)-1β, and lipopolysaccharide (LPS), upregulate PAR2 expression in the endothelial cells (10). The cleavage of

Correspondence to: Dae-Ki Kim, Department of Immunology, Chonbuk National University Medical School, 664-14 Deokjin-dong 1-ga, Deokjin-gu, Jeonju 561-756, Korea. (Tel) 82-63-270-3080, (Fax) 82-63-274-9833, (E-mail) deakim@chonbuk.ac.kr

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PAR2 by trypsin and tryptase can regulate the activities of inflammatory cells including endothelial cells, epithelial cells, smooth muscle cells and macrophages in a variety of tissues. PAR2 agonists are able to regulate the expression and release of inflammatory mediators such as cytokines, prostanoids, neuropeptides, and NO (11). Moreover, i.p. administration of PAR2-activating peptides induced leukocyte rolling, adhesion, proliferation, and extravasation through endogenous production of platelet-activating factor (12-14). Thus, there is evidence that stimulation of PAR2 promotes inflammatory responses in the tissues.

It has been reported that the cleavage of PAR2 also was involved in the activation and functioning of immune and inflammatory cells, such as neutrophils, eosinophils, and T cells (15-17). A recent study has shown that PAR2 was expressed in human macrophages, but not monocytes (18). However, the role of PAR-2 activation in human macrophages remains to be studied. Considering the significance of macrophages for pathological processes, such as inflammation, we have investigated the expression and functional activity of PAR-2 in macrophages. In this study, we confirmed the expression of PAR2 in human macrophages cell line THP-1 by RT-PCR and flow cytometry. We subsequently examined the effects of PAR2 agonist, trypsin and PAR2-activating peptide on the cell proliferation and functions of macrophage.

Materials and Methods

Cell culture and reagents. Human monocytic macrophage cell line, THP-1 (Korean Cell Line Bank, Korea) was maintained in DMEM supplemented with 10% fetal calf serum (FCS), penicillin, and streptomycin sulfate. Trypsin and soybean trypsin inhibitor (SBTI) were purchased from Sigma (St. Louis, MO). PAR2-activating peptides (AP) (SLIGKV-NH_2) and control peptide (LSIGKV-NH_2) (15) were purchased from Bachem (Torrence, CA). The specific monoclonal antibody against human PAR2 and control IgG were from Santa Cruz Biotechnology (Santa Cruz, CA).

Reverse-transcription PCR. Total RNA from THP-1 cells was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA). For cDNA synthesis, total RNA was denatured and reverse transcribed in a reaction buffer containing oligo (dT) and AMV reverse transcriptase (iNtRON, Korea) for 1 h at 42°C. PCR amplification (94°C for 30 sec, 55°C for 30 sec, and 72°C for 1 min; 30 cycles) was performed in a Perkin-Elmer thermal cycler (Norwalk, CT). The PCR-amplified samples were run on 1% agarose gel and visualized using ethidium bromide. Primers used in this study were as follows (17): PAR2 (sense, 5'-TGG ATG AGT TTT CTG CAT CTG TCC and antisense, 5'-CGT GAT GTT CAG GGC AGG AAT G), PAR4 (sense, 5'-AAC CTC TAT GGT GCC TAC GTG C and antisense, 5'-CCA AGC CCA GCT AAT TTT TG), and G3PDH (sense, 5'-GTC AAC GGA TTT GGT CGT ATT and antisense, 5'-AGT CTT CTG GGT GGC AGT GAT). All the primers were synthesized at the Bioneer Co. (Korea).

Flow cytometry. Cells (5×10^5 cells) were washed once with 1×PBS containing 0.1% NaN_3 (PBS-N), and incubated in 100μl PBS-N containing control mouse IgG_2a or mouse anti-PAR2 monoclonal antibody (1 : 200) for 20 min on ice. After washing in PBS-N, cells were immunostained with FITC-conjugated goat anti-mouse IgG antibody (1 : 200; Santa Cruz Biotech) for 30 min at 4°C. The cells were then washed in PBS-N, resuspended in PBS-N containing 1% paraformaldehyde (PFA), and kept at 4°C in the dark until analyzed in a FACScan flow cytometer (BD Systems, Mountain View, CA).

Enzyme-linked immunosorbent assay (ELISA) for TNF-α. THP-1 cells were starved in serum-free medium for 24 h before experimentation to prevent any interference with serum factors. Cells (10^4 cells/well) were resuspended in 200μl of serum-free medium containing 0.2% BSA and seeded in 96-cluster wells (SPL, Korea). After the treatment of PAR2 agonists or trypsin (10μg/ml), cells were incubated for the indicated times. The supernatants were collected by centrifugation for measurement of TNF-α. The level of TNF-α was determined using a commercially available ELISA kit (R&D Systems, Minneapolis, MN), according to the manufacturer's instructions.

Cell proliferation assay. Determination of cellular proliferation was accomplished by using the 3-(4, 5-imethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay. Cells were seeded at 5,000 cells/well in 96-cluster wells (SPL, Korea) in 200μl of complete medium. Cells were incubated with or without PAR2 agonists up to 3 days in a time (0, 1, 2, or 3 days) and concentration (0, 0.1, 1, 10, or 50μg/ml) dependent manner. The viability of cells was measured by counting in a hemacytometer after staining with 0.4% trypan blue. Treatment with trypsin (100μM) revealed 99% viability of THP-1 cells. After 24 h of incubation, 10μl of MTT (5 mg/ml) was added to each well and cultured for another 4 h at 37°C under 5 % CO_2. The supernatants were discarded and 100μl DMSO was added to each well to dissolve the produced formazan crystals. The optical density of formazan was measured at 540 nm using an ELISA reader. The mean value of three wells was calculated for cell proliferation in percentage values of control samples.

Western blot for ERK activation analysis. THP-1 cells stimulated with trypsin were washed with ice-cold

PBS twice and with ice-cold lysis buffer (iNtRON Biotech, Korea) for 20 minutes. Cell lysates were collected by centrifugation at 12,000 rpm for 15 min at 4°C. Total protein concentration was measured using a BCA protein assay reagent (Sigma). Proteins were separated by SDS-PAGE with 10% acrylamide gel and transferred to PVDF membrane (Invitrogen). After blocking with PBS containing 3% BSA, the membrane was immunobloted with anti-phospho-ERK1/2 (1 : 500) for 12 h at 4°C. HRP-conjugated antibody against rabbit IgG (1 : 2,000; Santa Cruz Biotech) was used as a secondary antibody. Finally, epitopes on proteins recognized specifically by antibodies were visualized by using enhanced chemiluminescence (ECL) detection kit (Amersham, Milan). After stripping, the membranes were reprobed with anti-ERK antibody as respective loading controls.

Statistical analysis. Data from at least three experiments using different cell preparations were summarized and represented as mean±S.E. Statistical analyses were performed using Student's *t*-test.

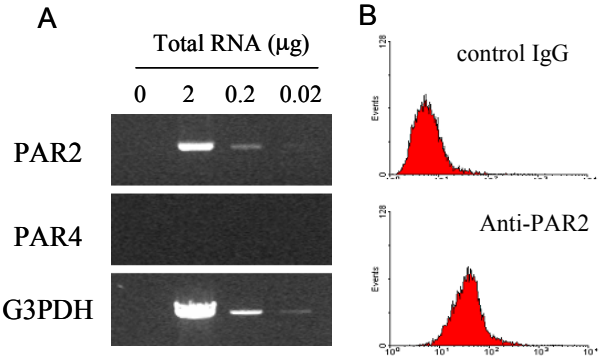

Figure 1. The expression of PAR2 mRNA and protein in human macrophage cell line THP-1. (A) Total RNA (0.02, 0.2, or 2μg) was reverse transcribed to cDNA and amplified by RT-PCR for the detection of PAR2 and PAR4 mRNA. G3PDH mRNA was used as control. (B) To determine the cell surface expression of PAR2 protein. THP-1 cells were immunostained with anti-PAR-2 or control IgG as negative control. PAR2-positive cells were detected by flow cytometry.

Results

Expression of PAR2, but not PAR4, in THP-1 cells. Recent study has shown that PAR2, but not PAR4, is expressed in human macrophages (18). To investigate the roles of PAR2 in human macrophage cell line THP-1, trypsin was used as a PAR2 agonist. Since typsin has been known to activate PAR2 and PAR4, we first confirmed the expression of PAR2 and PAR4 mRNA. As shown in Fig. 1A, RT-PCR analyses revealed that THP-1 cells express PAR2 mRNA, but not PAR4 mRNA. In addition, we observed the expression of PAR2 protein in THP-1 cells by flow cytometry with anti-PAR2 Ab (Fig. 1B). These results indicate that PAR2 is expressed on the surface of THP-1 cells.

Effects of PAR2-mediated activation on the proliferation of THP-1. To examine whether trypsin can act as a mitogen for human macrophages, we measured the cell numbers 48h after incubation with various concentrations of trypsin in THP-1 cells. As shown in Fig. 2A, the proliferation of THP-1 cells was increased concentration-dependently by stimulation with trypsin at concentrations up to 50μg/ml. Trypsin (10μg/ml) also induced the gradual increase in cell proliferation in a time-dependent manner (Fig. 2B). This increase was remarkably inhibited by the addition of 10μg/ml soybean trypsin inhibitor (SBTI) (Fig. 3). Moreover, we examined the effect of PAR2-activating peptides,

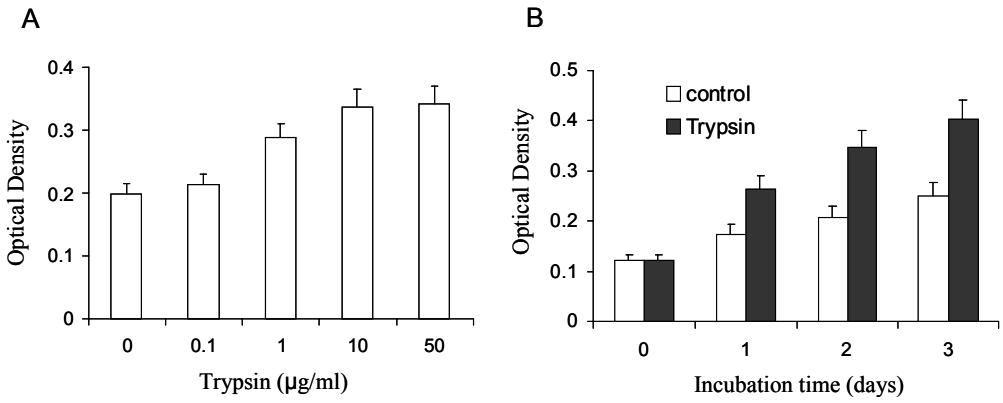

Figure 2. The effect of trypsin on the proliferation of THP-1 cells. (A) Cells were incubated for 48 h at a various concentration of trypsin. MTT was added to culture medium 4 h before cell harvest, and the optical density of formazan produced in cells was measured at 540 nm using an ELISA reader. (B) Cells were incubated with trypsin (10μg/ml) for the indicated time up to 3days. The proliferation activity was measured by MTT assay. Values are mean±S.E. of three experiments ($p < 0.05$).

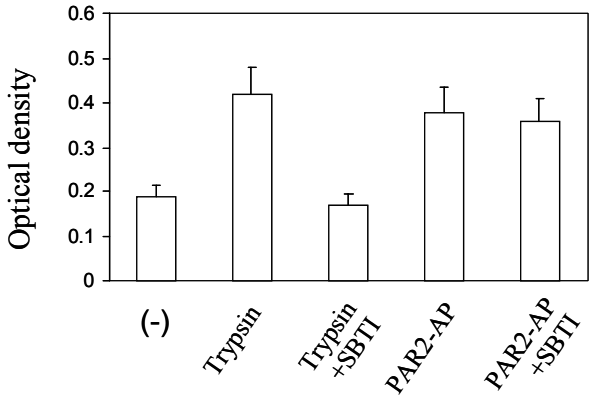

Figure 3. The inhibitory effect of soybean trypsin inhibitor (SBTI) on the PAR2-mediated proliferation in THP-1 cells. Cells were preincubated with or without SBTI (20μg/ml) for 30 min, and than stimulated with 10μg/ml of typsin or 20μM PAR2-activating peptide (PAR2-AP), SLIGKV. After stimulation for 48 h, the proliferation activity was measured by MTT assay. Values are means±S.E of three experiments ($p < 0.05$).

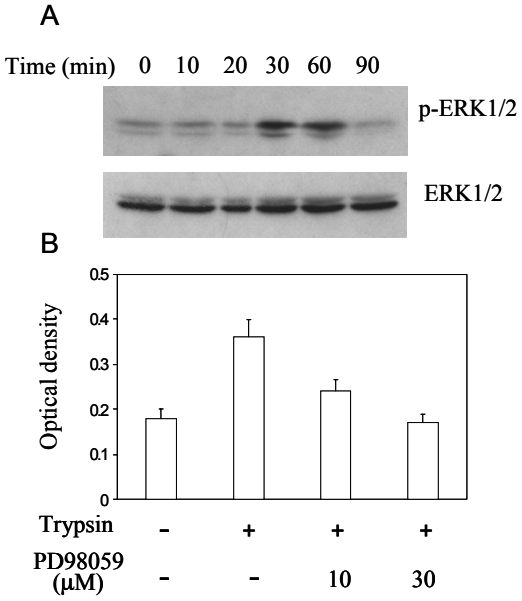

Figure 4. The induction of ERK phosphorylation in trypsin-stimulated THP-1 cells. (A) To determine ERK phosphorylation, cells were incubated with trypsin (10μg/ml) for times indicated and lysates were used for the western blot analysis with anti-p-ERK1/2 or anti-ERK1/2. (B) To examine the effect of ERK inhibitor (PD98059) in trypsin-induced cell proliferation, cells were pretreated with or without PD98059 (10 or 30μM) 10 min before stimulation with trypsin. After stimulation for 48 h, the proliferation activity was measured by MTT assay. Values are means±S.E of three experiments ($p < 0.05$).

SLIGKV, in THP-1 cells. We observed that cell proliferation activity induced by SLIGKV was similar to the response to trypsin. However, the cell proliferation induced by SLIGKV was not affected by the pretreatment with 10μg/ml SBTI (Fig. 3), indicating that SBTI causes blockage of trypsin proteolytic activity.

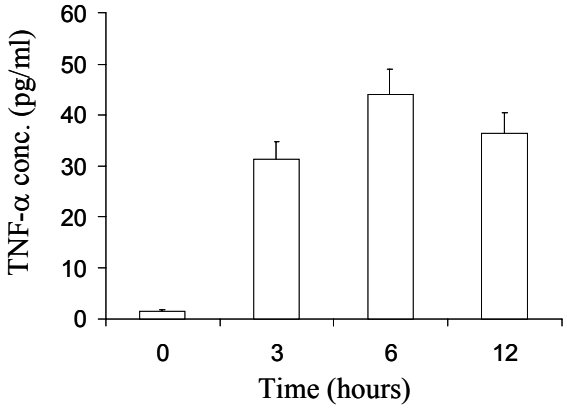

Figure 5. The effect of trypsin on the production of TNF-α in THP-1 cell. Cells were incubated in the presence of trypsin (10μg/ml) for times indicated and the supernatants were collected to measure the level of TNF-α using ELISA kit. Values are means±S.E of three experiments ($p < 0.05$).

Effect of trypsin on phosphorylation of ERK. We examined whether trypsin can affect the signaling pathway involved in cell proliferation in THP-1 cells. We therefore measured the cellular ERK activity after stimulation with trypsin (10μg/ml). Trypsin-induced ERK phosphorylation was peaked at 30 min (Fig. 4A). To examine whether activation of ERK is required for cell proliferation induced by trypsin, the cells were treated with ERK inhibitor, PD98059 (10 or 30μmol), before stimulation with trypsin (10μg/ml). As shown in Fig. 4B, pretreatment of the cells with PD98059 inhibited trypsin-induced cell proliferation and ERK activation. indicating that the trypsin-induced proliferation in THP-1 cells is dependent on ERK.

Effect of trypsin on TNF-α secretion by THP-1 cells. Next, to examine the functional activity of PAR2 in trypsin-treated THP-1 cells, we determined the secretion of TNF-α after stimulation with trypsin by ELISA. As shown in Fig. 5, stimulation with trypsin (10μg/ml) induced significantly the secretion of TNF-α from THP-1 cells. The secretion of TNF-α was peaked at 6h after stimulation with trypsin. In addition, we examined the effect of PAR2-activating peptides, SLIGKV, in THP-1 cells and found that the secretion of TNF-α by PAR2-AP (SLIGKV) was consistent with that by trypsin (data not shown). It suggests that PAR2 agonist potently stimulate human THP-1 cells to induce production of proinflammatory cytokines.

Discussion

In this study, we showed that trypsin could induce cell proliferation and phosphorylation of ERK of human macrophage cell line THP-1. We also showed that a trypsin inhibitor (SBTI) and ERK inhibitor inhibited trypsin-induced cell activation. It indicates that trypsin-induced cell proliferation is dependent on activation of ERK in a PAR2-dependent manner. In addition, we showed that activation of PAR2 results in a series of events including the release of TNF-α in THP-1 cells. These data represent that the cleavage of PAR2 by its physiological activators, trypsin-like serine proteases, might be a initiating event to control macrophage activation, suggesting a role of trypsin and PARs in macrophage cell proliferation.

It has been known that human macrophages are activated by other cysteine proteases, thrombin, through PAR1and PAR3 (19) in a PAR2-independent manner. Thrombin or thrombin-like serine proteases are most likely critical in coagulation. In contrast, trypsin-like serine proteases may activate inflammatory cells through PAR2 and may be involved in immunity and inflammation (20,21). It has recently been reported that thrombin and PAR1-AP induce IL-8 production and proliferation through the increased cytosolic Ca^{2+} in human monocytic U937 cells (22). As calcium signaling has been involved in the regulation of a variety of cellular responses including adhesion, cell motility, gene expression, and proliferation (23), It supports that PAR2 activation may results in the increased cytosolic Ca^{2+} in human macrophages. Therefore, it will be important to define the full repertoire of PARs and proteases that signal through PARs in macrophages.

Many allergens (24) most likely have trypsin-like protease activities. Moreover, during an allergic response, mast cells release their granular contents that include serine proteases, such as tryptase (25). Once macrophages are recruited into an allergic inflammation site, these allergens or granule enzymes may directly activate macrophages through the interaction of their trypsin-like serine protease and PAR2 and may induce inflammatory mediator release from macrophages. Conversely, removal of protease activity from the sites of inflammation or blockade of PAR2 may benefit patients with allergic diseases.

In summary, we demonstrated that human macrophages are activated through cleavage of PAR2 exposed to trypsin-like serine proteases, resulting to release inflammatory mediators. However, the PAR2-mediated macrophage activation that links to the pathophysiological events needs to be further elucidated. Most of inflammatory cells or immune cells can be stimulated directly with serine proteases through PAR2.

Understanding of cellular and molecular regulatory mechanisms of PAR2-mediated events may provide a new therapeutic insight for the management of inflammatory disorders.

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