Mast Cell Tryptase Stimulates DLD-1 Carcinoma Through Prostaglandin- and MAP Kinase-Dependent Manners

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Abstract. We found that striptease-positive mast cells were abundant in the invasive front of human colon adenocarcinoma by examining 30 cases. Because tryptase has been suggested to be the agonist proteinase for protease-activated receptor-2 (PAR-2), we investigated the effects of stimulation of PAR-2 by tryptase on the cell signaling and proliferation of DLD-1, a human colon carcinoma cell line. PAR-2 stimulation by tryptase induced the increase in \([\text{Ca}^{2+}]_i\), which was desensitized by the prior application of PAR-2 activating peptide (AP). The proliferative responses of DLD-1 to tryptase and PAR-2 AP were associated with the phosphorylation of MEK and MAP kinase. Inhibition of MEK by PD98059 completely inhibited the proliferation-enhancing effects of tryptase and PAR-2 AP as well as phosphorylation of MAP kinase. Moreover, tryptase and PAR-2 AP stimulated the production of prostaglandin E2 and the inhibition of prostaglandin synthesis by indomethacin or NS398 resulted in the complete inhibition of the proliferative responses to tryptase and PAR-2 AP. Furthermore, the tryptase-stimulated proliferation of DLD-1 was concentration-dependently inhibited by nafamostat mesilate, a specific inhibitor of tryptase. These results as a whole indicated that tryptase has proliferative effects on DLD-1 through cyclooxygenase- and MAP kinase-dependent manners acting on PAR-2 by its proteolytic activity.

Keywords: protease-activated receptor-2, MAP kinase, colon cancer, MEK, tryptase

Introduction

Protease-activated receptors (PARs) constitute a unique branch of the G-protein-coupled receptor superfamily. The PARs appeared to be activated by the cleavage of the N-terminal extracellular portion of the receptor by specific proteinases and the newly revealed N-terminal sequence, five to six amino acids tethered peptide, in turn can bind on the receptor as the tethered ligand, leading to the G-protein coupled signal transduction (1 – 5).

PAR-2, highly expressed in the gastrointestinal tract, has been demonstrated to be involved in the secretion and movement of the alimentary tract (6 – 8). In the previous study, we found that the stimulation of PAR-2 by activating peptide induced the proliferative response of DLD-1 human colon carcinoma (9). Thus, PAR-2 may be involved in the control of enterocytes in human colon. Several serine proteinases such as trypsin or trypsin-like proteinase have been suggested to be candidates for PAR-2 agonist proteinase. Among them, mast cell tryptase was reported to be an agonist proteinase for PAR-2 in vascular endothelial cells (10, 11). Mast cells are present in mucosa and submucosal tissue of the gastrointestinal tract and there have been many reports suggesting the involvement of mast cells in the growth of tumor tissues through the stimulation of angiogenesis by the active substances released from mast cells (12 – 15). Therefore, in the present study we first investigated the distribution of tryptase-positive mast cells in human colon cancer tissues from thirty cases. Then, we examined the direct effects of tryptase...
on the proliferation and intracellular signaling in DLD-1 carcinoma cells in vitro. We found that tryptase-positive mast cells were abundant specifically in the invasive front of colon cancer tissues and that the stimulation of DLD-1 cells by tryptase induced PAR-2-mediated proliferation in MAP kinase- and cyclooxygenase (COX)-dependent manners. These results suggested the tumor growth-promoting effects of tryptase released from mast cells, which might be regulated by specific trypsin inhibitors.

**Materials and Methods**

All experiments were performed according to Ethical Guidelines of Okayama University Graduate School of Medicine and Dentistry and approved by Institutional Research Board.

**Reagents**

The human PAR peptides were purchased from Bio Synthesis (Nagoya). The activating and inverse peptides used in the present study were PAR-1 activating peptide (AP), SFLLRN; PAR-1 inverse peptide (IP), FSLLRN; PAR-2 AP, SLIGKV; and PAR-2 IP, LSIGKV.

Human tryptase, Fluo-3AM, human thrombin, PD98059, indomethacin, and NS398 were from Sigma (St. Louis, MO, USA).

**Immunohistochemical staining**

Human colon cancer tissues were fixed with 10% formalin in 0.1 M phosphate-buffered saline immediately after the surgical dissection. The fixed tissues were dehydrated through an ethanol series, embedded in paraffin, and cut into 5 – 7-µm thin sections. After blocking with 10% normal goat serum, mouse monoclonal anti-human mast cell tryptase antibody (AA1) (Dako Cytomation, Kyoto) was applied to the sections for 24 h at 4°C. The sections were successively treated with biotinylated anti-mouse IgG goat IgG and horseradish peroxidase-conjugated streptavidin with washings in between. The reaction was developed by the addition of substrates, diaminobendazine and hydrogen peroxide. The cells were harvested with biotinylated anti-mouse IgG goat IgG and horseradish peroxidase-conjugated streptavidin with washings in between. The reaction was developed by the addition of substrates, diaminobendazine and hydrogen peroxide. The cells were harvested with biotinylated anti-mouse IgG goat IgG and horseradish peroxidase-conjugated streptavidin with washings in between.

**Cells and culture conditions**

DLD-1 cells were obtained from JCRB Cell Bank (Tokyo). The cells were cultured in RPMI-1640 supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 mg/ml streptomycin. This cell line was grown in monolayer.

**RT-PCR**

Total RNA was isolated from human colon cancer tissue and DLD-1 cells using RNAzolB (Cinna/bio Texc, Friedswood, TX, USA) in a single-step phenol-extraction method and used as templates. Reverse transcription was performed at 22°C for 10 min and then 4°C for 20 min using 1.0 µg of RNA. PCR was performed as described previously (9). The following specific primers were used: PAR-2 sense (5'-GGG CAC TCC AGG AAG GCA-3') and PAR-2 antisense (5'-TCA GGG ATC ATG CAA AGA-3'). The PCR amplification consisted of 35 cycles of 30 s at 95°C, 30 s at 64°C, and 2 min at 72°C using Taq DNA polymerase. The PCR products were resolved on 1% agarose gels and visualized by ethidium bromide staining.

**Cytosolic Ca²⁺ measurements**

The free intracellular Ca²⁺ concentration ([Ca²⁺]) was determined using the Ca²⁺ sensitive fluorescent dye Fluo3-AM as described previously (9). DLD-1 cells were cultured on a glass bottom culture dish (35 mm in diameter; MatTek, Ashland, MA, USA). For dye loading, medium was replaced with RPMI-1640 containing 10 µM Fluo3-AM (Sigma), incubated for 60 min at 37°C, and washed twice with the buffer for measuring Ca²⁺ signal. The Ca²⁺ signal was measured 30 min after the culture had been kept at 25°C. The calcium measuring buffer has the following composition: 115 mM NaCl, 5.0 mM KCl, 2.5 mM CaCl₂, 0.4 mM KH₂PO₄, 0.4 mM Na₂HPO₄, 20.0 mM HEPES, 10.0 mM glucose, pH 7.4 adjusted with tris(hydroxymethyl)amino-methane. Fluorescence measurements of [Ca²⁺], were performed using an confocal microscopy system (LSM-510; Zeiss, Oberkochen, Germany), excited at 488 nm and detected at 505 – 550 nm band width.

**Cell growth assay**

DLD-1 cells were plated in pentaplicate at a density of 5.0 × 10⁴ cells/25 ml flask. One day later, medium was changed with RPMI-1640 including PAR agonist proteinase or activating peptide. The cells were harvested by the treatment with trypsin/EDTA and the cell number was counted under light microscopy at the indicated days after viability was determined by trypan blue exclusion.

**Immunoblotting**

Immunoblotting of protein extracts from human colon cancer tissues and DLD-1 cells was performed. The cancer tissue proper and the normal mucosa at least 2 cm distant from the invasive edge were prepared from a freshly dissected human colon cancer specimen by homogenizing tissues in 50 mM tris-HCl buffer contain-
After determining the protein content, the homogenates from colon tissues and DLD-1 cells were solubilized in sodium dodecyl sulfate (SDS) sample buffer (125 mM Tris pH 6.8, 4% SDS, 20% glycerol). After SDS-PAGE, the proteins were transferred onto the nitrocellulose membranes and were blotted with antibodies against PAR-2 (Santa Cruz Biotechnology), MEK, MAP kinase, and their phosphorylated forms (Cell Signaling Technology, Beverly, MA, USA). The reaction was developed as described previously (9, 16).

**Determination of prostaglandin (PG) E \(_2\)**

For PGE\(_2\) determination, the cells (5 \(\times\) 10\(^3\)) were precultured in RPMI-1640 with 10% FCS for 96 h in a 96-well plate and then were incubated with agonists for the periods indicated. PGE\(_2\) in the culture media was determined by PGE\(_2\) EIA Kit-monoclonal (Cayman Chemical, Ann Arbor, MI, USA). The results were expressed as percent of the control in the presence of medium alone.

**Statistical examination**

The statistical significance of differences was evaluated by ANOVA followed by Dunnett’s test. A probability value less than 0.05 was considered significant.

**Results**

RT-PCR showed that PAR-2 mRNA was expressed in DLD-1 cells and colon cancer tissue (Fig. 1A). The expression of PAR-2 was also examined by Western blotting (Fig. 1B). It was revealed that PAR-2 protein in the adenocarcinoma was higher than that in the normal mucosa from the same patient (Fig. 1B). We observed similar results in three different cases. We then investigated the distribution of tryptase-positive mast cells as well as PAR-2 immunohistochemically in 30 cases of human adenocarcinomas (Fig. 2). Figure 2 shows the typical histological specimens of human colon adenocarcinoma. Apparently, tryptase-positive mast cells (brown color in Fig. 2B as indicated by arrowheads)
Fig. 3. Distribution of tryptase-positive mast cells in the normal colon tissue (A) and the invasive front of human colon cancer tissues (B – D). Letters T and N show the location of the tumor mass and normal mucosa, respectively. The tryptase-positive mast cells were stained immunohistochemically (brown color) and the sections were counterstained with hematoxylin. Sections A and B are from the normal mucosa and the invasive front of the colon cancer tissue of the same patient, respectively. C and D show the similar pattern of distribution of tryptase-positive mast cells in the specimen from another patient. Bars = 200 µm.

Fig. 4. The number of tryptase-positive mast cells was counted in the center of the colon cancer tissue (C), invasive front of cancer tissue (B), and normal area (N) at least 5 mm distant from the invasive edge. The mast cells were counted under ×400 magnification fields. The results are the means ± S.E.M. of 30 patients. **P<0.01. Bars = 200 µm.
were concentrated in the stroma of the invasive front of the tumor tissues. In contrast, the distribution of PAR-2 immunoreactivities was rather even throughout the tumor mass and the intensities of PAR-2 immunoreactivity in the tumor mass were higher than those in the normal mucosal area (Fig. 2C). This finding was consistent with the results obtained by Western blotting for PAR-2. In Fig. 3, numerous tryptase-positive mast cells were observed in the invasive front and the surrounding areas of adenocarcinoma. We compared the mast cell number in three regions: the center of the adenocarcinoma, the invasive front of adenocarcinoma, and the normal tissue area at least 5-mm distant from the invasive front. Figure 4 summarizes the quantitative analysis of mast cell numbers and revealed a remarkable contrast of the tryptase-positive mast cell distribution in three regions. In the normal tissue area, the average number of tryptase-positive mast cells was 20/field, whereas those in the invasive front was 4-fold that of the normal tissues. On the other hand, the number in the center of adenocarcinoma was 6 times less than those in the normal tissues. This specific pattern of distribution was consistent among the clinical cases examined.

Then, we used DLD-1 colon cancer cells to examine whether mast cell tryptase can stimulate PAR-2 because we found that DLD-1 cells expressed functional PAR-2 responding to PAR-2 AP in the previous study (9). Figure 5 shows the calcium signaling induced by PAR-1 AP (50 µM), PAR-2 AP (50 µM), and tryptase (1 nM). All three agonists produced a transient increase in intracellular Ca²⁺. As shown in Fig. 5, C and D, calcium signaling induced by PAR-2 AP was desensitized after the stimulation with tryptase (1 nM) and vice versa. On the other hand, there was relatively small desensitization between PAR-1 AP (50 µM) and tryptase (1 nM) (Fig. 5: A and B). These results strongly suggested that tryptase stimulated PAR-2 specifically.

Tryptase at 1 nM enhanced the proliferation of DLD-1 cells as did PAR-2 AP (50 µM) (Fig. 6). PAR-2 IP and PAR-1 agonists had no effect on DLD-1 proliferation. Next, we examined whether the phosphorylation of MEK1/2 and MAP kinases was induced by tryptase because we observed the PAR-2 AP-initiated phosphorylation of MEK1/2 and ERK1/2 in the previous study (9). Tryptase at 1 nM phosphorylated MEK1/2 and ERK1/2 within 10 min after the stimulation of DLD-1 cells (Fig. 7), but not JNK and p38 MAP kinase (data not shown). The inhibition of MEK by PD98059 (50 µM) completely inhibited the proliferation potentiating effects of tryptase (1 nM) and PAR-2 AP (50 µM) (Fig. 8) as well as the phosphorylation of ERK1/2 in DLD-1 cells (Fig. 7).

**Fig. 5.** Desensitization of Ca²⁺ signaling in DLD-1 cells between stimuli with tryptase and PAR-2 AP. DLD-1 cells were first stimulated by tryptase or PAR AP and then stimulated by PAR AP or tryptase, respectively. Ca²⁺ signaling in DLD-1 cells were detected as described in Materials and Methods. Typical results are shown.
Tryptase Stimulates DLD-1 Proliferation

PAR-2 AP (50 µM) and tryptase (1 nM) significantly stimulated the production of PGE\(_2\) in DLD-1 cells 10 min after the start of stimulation, and this effect was partially inhibited by PD98059 (50 µM) (Fig. 9). On the other hand, the inhibition of cyclooxygenase by indomethacin (30 µM), COX-1 and COX-2 non-selective inhibitor, and NS-398 (10 µM), COX-2 selective inhibitor, completely inhibited the proliferation stimulating effects of PAR-2 AP (50 µM) and tryptase (1 nM) (Fig. 10). This finding indicated that DLD-1-proliferating effects of PAR-2 stimulation was mediated by the production of PGs. Nafamostat mesilate was reported to be a very potent inhibitor of human tryptase (10, 11, 17). Therefore, we determined the antagonizing effects of nafamostat on tryptase-induced proliferation of DLD-1 cells. As shown in Fig. 11, nafamostat concentration-dependently inhibited the tryptase-induced but not PAR-2 AP-induced enhancement of proliferation of DLD-1 cells. Thus, the stimulation of PAR-2 by tryptase was demonstrated to be dependent on its proteolytic activity.

Discussion

In the present study, we found that the considerable number of mast cells were present in the invasive front of tumor tissue of human colon cancer cells. In contrast, there were few mast cells in the central part of the tumor tissues. We also clearly showed that tryptase stimulated...
the proliferation of DLD-1 cells through the stimulation of PAR-2 because the complete desensitization of calcium signaling was observed in DLD-1 cells between PAR-2 AP and tryptase. As observed in the previous study using PAR-2 AP (9), tryptase activated MAP kinase but not JNK and p38 kinase, and the proliferating effects were blocked by MEK inhibitor PD98059. Moreover, we found that the PAR-2 stimulation by tryptase or PAR-2 AP induce the production of PGE$_2$ and that the inhibition of COX by indomethacin and NS398 completely inhibited the proliferative response of DLD-1 cells. On the other hand, PD98059 only partially inhibited PGE$_2$ production, suggesting that both MAP kinase activation and PG synthesis was prerequisite for DLD-1 proliferation and that PGE$_2$ production alone was not enough for DLD-1 proliferation. It was concluded that the ERK signaling and the action of PGE$_2$ was important for the proliferation-enhancing effect mediated by PAR-2, and it was suggested that both signalings may be interconnected each other. In the present study, we could not determine PGE$_2$ levels in the medium after prolonged incubation with PAR-2 agonists. This might provide additional information on PAR-2-mediated activation of ERK and PGE$_2$ production. Further work is necessary to clarify this issue.

The immunohistochemical study revealed that the density of PAR-2 immunoreactivities in tumor tissues were higher than those in the normal tissues. Thus, tryptase released from mast cells present in the invasive front of tumor tissues may stimulate the PAR-2 on the
colon cancer cells in a paracrine manner, leading to the proliferation of colon cancer cells. There have been cumulative evidences supporting that mast cells are involved in angiogenesis through the release of plural angiogenic substances such as vascular endothelial growth factor and angiopoietin (13, 18) and that the angiogenic response to these substances released from the mast cells surrounding tumor tissues promotes the growth of tumor tissues (19 – 22). Therefore, the mast cells migrating to and accumulating around the cancer tissues may support the growth of cancer cells indirectly through the stimulation of angiogenesis as well as the direct growth promotion via the stimulation of PAR-2. The stimulation of PAR-2 on vascular endothelial cells was also reported to be involved in angiogenesis (23, 24). These findings as a whole suggest that mast cells may support colon cancer tissues in two ways: direct proliferation via PAR-2 stimulation and indirect support through angiogenesis.

One of the important findings in the present study was that PAR-2-mediated proliferation of DLD-1 was COX-dependent. In the previous study, we observed the exposure duration-dependent effect of PAR2-AP on DLD-1 proliferation (9). Because the PGE$_2$ production appeared to be time-dependent, PGE$_2$ may be a missing factor in the previous study for the time-dependent effect of PAR-2 stimulation on DLD-1 proliferation (9). Recently, Pozzi et al. (25) also reported that PGE$_2$ stimulated the proliferation of mouse colon adenocarcinoma (CT26) through EP$_4$ prostanoid receptors. Thus, PGE$_2$ may be an important mediator in the stimulation of colon cancer cells. Clinical trials revealed the preventive effects of long term treatment with COX inhibitors on the incidence of colon cancer cells (26 – 29). PGs produced by COX has been suggested to be involved in tumor growth via different mechanisms: direct stimulation of cancer cell growth, stimulation of angiogenesis, and immunosuppression (29). If PAR-2-mediated production of PGs constitutes a considerable portion of PG synthesis in the colon cancer tissue, the regulation of PAR-2 stimulation would be a new therapeutic target of colon cancer. Nafamostat mesilate was demonstrated to be a very potent inhibitor of human tryptase (11, 17). In the present study, nafamostat mesilate concentration-dependently inhibited the tryptase-induced proliferation of DLD-1 cells. Thus, tryptase may be a novel target of colon cancer therapy. Plural trypsin-like serine proteinases have been cloned from colon cancer cell lines (30 – 32). Thus, an autocrine mechanism for the stimulation of PAR-2 may also exist in colon cancer tissue in addition to the paracrine mechanism triggered by tryptase.

In conclusion, we demonstrated the specific localization of tryptase-positive mast cells in the invasive front of human colon cancer. The analysis of in vitro proliferative effects of tryptase on a colon cancer cell line, DLD-1 cells, confirmed that tryptase directly activated PAR-2, leading to the ERK- and PG-dependent proliferation. Further research using different colon cancer cell lines are necessary to generalize the present findings.

References


