The Role of Mast Cell Tryptase in Regulating Endothelial Cell Proliferation, Cytokine Release, and Adhesion Molecule Expression: Tryptase Induces Expression of mRNA for IL-1β and IL-8 and Stimulates the Selective Release of IL-8 from Human Umbilical Vein Endothelial Cells

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The Role of Mast Cell Tryptase in Regulating Endothelial Cell Proliferation, Cytokine Release, and Adhesion Molecule Expression: Tryptase Induces Expression of mRNA for IL-1β and IL-8 and Stimulates the Selective Release of IL-8 from Human Umbilical Vein Endothelial Cells

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Mast cells are found frequently in close proximity to blood vessels, and endothelial cells are likely to be exposed to high concentrations of their granule mediators. We have investigated the proinflammatory actions of the major mast cell product tryptase on HUVEC. Addition of purified tryptase was found to stimulate thymidine incorporation, but induced little alteration in cell numbers, suggesting it is not a growth factor for HUVEC. Expression of ICAM-1, VCAM-1, and E-selectin was not altered following incubation with tryptase, but the potent granulocyte chemoattractant IL-8 was released in a dose-dependent fashion in response to physiologically relevant concentrations, with maximal levels in supernatants after 24 h. The actions of tryptase on HUVEC were inhibited by heat inactivation of the enzyme, or by preincubating with the protease inhibitors leupeptin or benzamidine, suggesting a requirement for an intact catalytic site. Reverse-transcription PCR analysis indicated up-regulation of mRNA for IL-8 as well as for IL-1β in response to tryptase or TNF-α. However, tryptase was a more selective stimulus than TNF-α and did not induce increased expression of mRNA for granulocyte-macrophage CSF or stimulate the release of this cytokine. Leukocyte accumulation in response to tryptase may be mediated in part through the selective secretion of IL-8 from endothelial cells. The Journal of Immunology, 1998, 161: 1939–1946.

Some of the earliest investigations of the role of tryptase focused on its ability to cleave certain extracellular substrates, including vasoactive intestinal peptide and calcitonin gene-related peptide (7, 8), 72-kDa gelatinase, fibronectin (9), prostromelysin (10), and kininogens (11). More recently, it has been established that tryptase can also alter cell behavior. For example, tryptase is a potent growth factor for a number of cell types, including fibroblasts (12, 13), epithelial cells (14), and airway smooth muscle cells (15), and is able to stimulate vascular tube formation in dermal microvascular endothelial cells (16). In fibroblasts, tryptase can also induce type I collagen synthesis and cell chemotaxis (13, 17). Of particular importance to an understanding of the contribution of tryptase in inflammation is the observation that this protease can stimulate release of the granulocyte chemoattractant IL-8 and up-regulate expression of ICAM-1 on H292 epithelial cells (14). Nevertheless, the potential proinflammatory actions of tryptase on endothelial cells have not been investigated.

No longer regarded simply as a passive barrier separating the blood and surrounding tissue, endothelial cells are now recognized as key players in the process of inflammation (18). The strategic positioning of the endothelium between the blood and tissue allows it to regulate the flow of inflammatory cells to and from a site of inflammation. Activation of the endothelium by proinflammatory cytokines such as TNF-α or IL-1 can stimulate the up-regulation of various cell surface adhesion molecules, including ICAM-1, VCAM-1, and E-selectin (19–22), promoting the adherence of inflammatory cells to the vessel wall before they migrate into the tissue. Moreover, endothelial cell activation by TNF-α can result in the production and release of potent inflammatory mediators that can facilitate the recruitment and activation of granulocytes (23). For example, IL-8 production by endothelial cells can...
promote neutrophil accumulation (23), and GM-CSF\(^3\) release from endothelial cells can stimulate the maturation and activation of granulocytes (24). It has been reported recently that thrombin, a protease with structural similarities to tryptase, can induce the release of IL-8 and up-regulate the expression of E-selectin on endothelial cells (25).

We have investigated the ability of tryptase to stimulate the proliferation of HUVEC. induce cytokine production, and alter adhesion molecule expression. We report that this major mast cell product has proinflammatory actions on endothelial cells and can induce expression of mRNA for IL-1\(\beta\) and IL-8 and the selective release of IL-8.

Materials and Methods

Materials and reagents

Heparin agarose, Sepharyl S-200, collagenase type I A, N-α-benzoyl-\(\text{L-arginine-}\)p-nitroanilide hydrochloride (BAPNA), leupeptin, benzamidine hydrochloride, bovine lung heparin glycosaminoglycan, gentamicin, 2% gelatin solution, avidin peroxidase conjugate, nonenzymatic cell dissociation fluid, and BSA were purchased from Sigma (Poole, Dorset, U.K.); anti-platelet endothelial cell adhesion molecule 1 Ab and anti-mouse FICT-conjugated Ab from Dako (High Wycombe, Bucks, U.K.); anti-EN4 Ab from Charles River Endosafe (Bognor Regis, West Sussex, U.K.); and electrophoresis grade agarose from Bio-Rad Laboratories (Hemel Hempstead, Herts, U.K.); anti-ICAM-1 Ab, anti-VCAM-1 Ab, and anti-E-selectin Ab from Serotec (Kidlington, Oxford, U.K.); rTNF-α and endothelial cell growth factor (ECGF) from R&D Systems (Abingdon, U.K.); methyl-[\(\text{H}\)]thymidine from Amersham (Little Chalfont, Bucks, U.K.); Affi-prep polyvinyl B matrix, silver staining kit, and electrophoresis grade agarose from Bio-Rad Laboratories (Hemel Hempstead, Herts, U.K.); paired Abs specific for GM-CSF from Pharmingen (Cambridge, U.K.); RGM-CSF, reverse-transcriptase system, and the cell proliferation assay from Promega (Southampton, U.K.); IL-8 and IL-1\(\beta\) (precursor) ELISA kits from Eurogentec (Teddington, U.K.); IL-1\(\beta\) (natural) ELISA kit from Genzyme (Cambridge, U.K.); the H292 epithelial cell line, derived from human lung mucoepidermoid carcinoma, from European Collection of Animal Cell Cultures (Porton Down, Wiltshire, U.K.); endothelial basal medium (EBM), epidermal growth factor, bovine brain extract, hydrocortisone, amphotericin, and FCS from Clonetics (Bucks, U.K.); Trizol, trypsin/EDTA solution, and RPMI 1640 medium from Life Technologies (Paisley, Scotland, U.K.); filters from Amicon (Stonehouse, Gloucestershire, U.K.); Coomassie blue protein assay from Pierce (Chester, U.K.); the Limulus amebocyte lysate endotoxin assay kit from Charles River Endosafe (Bognor Regis, West Sussex, U.K.); and adenine phosphoribosyltransferase (APRT), GM-CSF, IL-1\(\beta\), and IL-8 primers were synthesized by the Department of Microbiology, Southampton General Hospital (Southampton, U.K.).

Purification of tryptase

Lung tissue obtained postmortem (approximately 400 g) was chopped finely, homogenized, and extracted, as described previously (13, 26). Briefly, the tissue was washed in a low salt buffer before extraction in a high salt buffer and subjected to heparin agarose affinity chromatography. Bound protein was eluted over a salt gradient of 0.4 M to 1.2 M NaCl, and fractions containing tryptic activity (eluting at approximately 0.8 M NaCl) were pooled, diluted to 0.4 M NaCl, and reapplied to the column for a second time. Fractions with tryptic activity were pooled and concentrated, sterile filtered, and stored at -20°C in aliquots of approximately 1 to 3 U/ml.

\(^3\) Abbreviations used in this paper: GM-CSF, granulocyte-macrophage CSF; APRT, adenine phosphoribosyltransferase; BAPNA, N-\(\text{Benzoyl-}\)L-\(\text{Arginine-}\)p-nitroanilide; EBM, endothelial basal medium; ECGF, endothelial cell growth factor; MTT, 3-(4, 5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide; PAR, protease-activated receptor.

Assessment of tryptase activity and purity

An aliquot of 90 µl tryptase assay buffer (100 mM Tris base, 1 M glycerol, pH 8) containing 1 mM BAPNA was added to 10 µl tryptase sample, and the initial reaction was monitored spectrophotometrically at 450 nm on an ELISA plate reader. A unit of tryptase activity was defined as that required to hydrolyse 1 µmol of BAPNA/min at 25°C. The purity of the isolated tryptase was assessed by SDS-PAGE on a 12% gel, staining with a silver staining procedure. To confirm the identity of the purified protein as tryptase, Western blotting was performed with the mouse anti-tryptase mAb AAs5 (27) and an anti-mouse IgG Ab conjugated to horseradish peroxidase. To further confirm the purity and identity of the protein as tryptase, the band on a blot was subjected to amino acid sequence analysis (Molecular Biology Unit, University of Newcastle, U.K.).

Cell culture

Endothelial cells were cultured from human umbilical cord veins by a method described previously (28), with slight modifications. Cells were grown and propagated on gelatin-coated flasks in EBM, supplemented with 2% FCS, 1 µg/ml hydrocortisone, 10 ng/ml epidermal growth factor, 50 µg/ml gentamicin, 50 ng/ml amphotericin B, and bovine brain extract. The following day, the cells were washed three times with PBS before the addition of fresh medium. Cultures had the typical cobblestone morphology of confluent endothelial cell monolayers, and the purity was consistently greater than 99%, as assessed by immunohistochemistry with an Ab specific for the endothelial cell surface marker EN4 or the cell adhesion molecule platelet endothelial cell adhesion molecule 1. Cells were used at passages 1 to 2 for all experiments. The H292 epithelial cell line was grown in RPMI 1640 medium, supplemented with 10% FCS and 50 µg/ml gentamicin. Both HUVEC and epithelial cells were maintained at a temperature of 37°C in a humidified atmosphere of 95% air and 5% CO\(_2\).

Assays for mitogenesis

Three different techniques were used to investigate tryptase as a growth factor for HUVEC: measurement of thymidine incorporation, direct cell counting, or application of a cell proliferation assay in which cell numbers are assessed by monitoring the conversion of the tetrazolium salt 3-(4, 5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to a blue formazan product by mitochondorial dehydrogenase in viable cells (MTT assay). For the thymidine incorporation assay, HUVEC in full medium were seeded into a 96-well microtiter plate (Becton Dickinson, Plymouth, UK). Cell density of 150 cells/mm\(^2\) and allowed to adhere for 24 h. The medium was replaced with serum-free medium (1 µg/ml insulin, 5 µg/ml transferrin, 5 µg/ml selenium, EBM, and 50 µg/ml gentamicin) for a period of 24 h, and the cells were incubated with either tryptase at concentrations ranging from 5 to 100 mM/U/ml, or ECGF at 1.6 ng/ml (a concentration found to be optimal in preliminary experiments). To determine the degree of dependency on an active site, tryptase was incubated with 20 µg/ml leupeptin on ice or heat treated at 56°C for 1 h before being incubated with cells, and the degree to which enzymatic activity was inhibited by these various treatments was assessed by monitoring the cleavage of BAPNA. Following a 24-h incubation, 1 µCi/well of methyl-[\(\text{H}\)]thymidine was added for an additional 8 h, and the cells were harvested on a 0.7-µm-pore glass fibre filter (GF/F filter) and counted in scintillant.

In separate experiments, HUVEC were plated at 150 cells/mm\(^2\) in a six-well culture plate. After allowing the cells to quiesce in serum-free conditions for 24 h, either tryptase (20 mM/U/ml) or ECGF (1.6 ng/ml) was added and incubated for an additional 8 h. Cells were counted using a modified Neubauer hemocytometer. The MTT cell proliferation assay was performed according to the manufacturer’s protocol (Promega). Briefly, HUVEC were plated into a 96-well microtiter plate at 300 cells/mm\(^2\) and left to reach confluence for 48 h. Cells were allowed to quiesce in serum-free medium before addition of test agents, and incubated for 48 h. To the cells, 15 µl of MTT dye solution was added and left for 1 h before the addition of 100 µl of stop/solubilization solution. The OD of individual wells was measured at 550 nm after an overnight incubation. Actual cell number was calculated from a standard curve obtained using known amounts of cells simultaneously plated as controls. Experimental conditions for epithelial cells were the same as those described for HUVEC. To allow direct comparison between these two cell types, we chose FCS (10%) as a positive control for proliferation in both cell lines and the fusion method involving incubation of cells in 0.04% trypan blue was utilized to assess potential cytotoxic actions of tryptase on HUVEC.
**Investigation of cytokine release**

HUVEC at a density of 500 cells/mm² were incubated in full medium and allowed to adhere in a 48-well tissue culture plate. Supernatants were removed and serum-free medium was added for 48 h. Tryptase at concentrations of 5 to 100 μM/ml or TNF-α (10 U/ml) was then added for an additional 24 h. Time-course experiments involved addition of a standard concentration of trypase (50 μM/ml) or TNF-α (10 U/ml) for up to 48 h. The protease inhibitors leupeptin (20 μg/ml) or benzamidine (15 μg/ml) were incubated with trypase preparations for 1 h on ice, or trypase was incubated at 56°C for 1 h before being added to the cells, and the inhibition of enzymatic activity was confirmed by measuring cleavage of BAPNA. In addition, certain samples were incubated overnight with a trypase-specific mAb AA5, before removal of the Ab/Ag complex by centrifugation, using protein A-Sepharose beads. The concentrations of IL-8, IL-1β (mature), IL-1β (precursor), and GM-CSF in supernatants were analyzed by ELISA according to the protocols of the manufacturers.

**Reverse-transcription PCR**

HUVEC were seeded at 500 cells/mm² in a 24-well plate before allowing to quiesce in serum-free medium for a period of 48 h. Tryptase (50 μM/ml) or TNF-α (10 U/ml) was incubated with the cells for 4 h (preliminary experiments showed this time point to be optimal for up-regulation of mRNA) before being lysed in the presence of Trizol and chloroform. The RNA was precipitated at −20°C in isopropanol overnight. The RNA pellet was recovered by centrifugation at 4°C, washed in 80% ethanol, air dried, and resuspended in diethyl-pyrocarbonate-treated water and quantified spectrophotometrically at 260 nm. One microgram of total cellular RNA was reverse transcribed by AMV reverse transcriptase at 42°C for 1 h using poly(dT)15 as a primer. The cDNA was amplified by PCR in the presence of a master mix containing the PCR buffer, MgCl₂, 1 U Taq DNA polymerase (Promega), 0.2 mM dNTPs, and specific primer pairs for either IL-8, sense primer 5’-GCA GCT CTG TGT GAA GGT GCA-3’ and antisense primer 5’-CAG ACA GAG CTC TCT TCC AT-3’; for GM-CSF, sense primer 5’-GCA GCT GAA TGC CAT CCA GG-3’ and antisense primer 5’-GCT TGT AGT GCC TGG CCA TC-3’; for IL-1β, sense primer 5’-AAC AGG CTG TGT GAA GGT GCA-3’ and antisense primer 5’-TAA GCC TCG TTA CCT CAT GT-3’; or APRT, sense primer 5’-GCT GCG TGC TCA TCC GAA AG-3’ and antisense primer 5’-GCC CCG AGA GTC TCT GCT C-3’. PCR was conducted for 40 cycles under the following conditions: denaturation at 94°C for 20 s, annealing at optimal temperature for each primer pair for 30 s, and extension at 72°C for 60 s in a thermocycler. Final extension was at 72°C for 10 min. PCR-amplified products (10 μl) were electrophoresed through 2% agarose gels containing 0.5 μg/ml ethidium bromide, and compared with DNA reference markers, visualizing products by UV illumination.

**Flow cytometry**

HUVEC at 500 cells/mm² in full medium were allowed to adhere to a 24-well tissue culture plate before supernatants were removed and replaced with serum-free medium. After allowing cells to quiesce for 48 h, trypase (5–100 μM/ml) or TNF-α (10 U/ml) was added for 4 h to study expression of E-selectin, 12 h for VCAM-1, and 24 h for expression of ICAM-1. The time points chosen have been reported to be optimal for the study of these adhesion molecules with other stimuli (19–22). In addition, time-course experiments involved addition of a standard concentration of trypase (50 μM/ml) or TNF-α (10 U/ml) for up to 48 h. The protease inhibitors leupeptin (20 μg/ml) or benzamidine (15 μg/ml) were incubated with trypase preparations for 1 h on ice, or trypase was incubated at 56°C for 1 h before being added to the cells, and the inhibition of enzymatic activity was confirmed by measuring cleavage of BAPNA. In addition, certain samples were incubated overnight with a trypase-specific mAb AA5, before removal of the Ab/Ag complex by centrifugation, using protein A-Sepharose beads. The concentrations of IL-8, IL-1β (mature), IL-1β (precursor), and GM-CSF in supernatants were analyzed by ELISA according to the protocols of the manufacturers.

**Results**

**Trypsin purification**

SDS-PAGE of the purified trypsin indicated a single diffuse band with a m.w. of 29 to 31 kDa, with no contaminants visible on silver-stained gels. Western blotting using the anti-trypsin mAb AA5 confirmed its identity as trypsin. N-terminal amino acid analysis of the first 12 amino acids revealed the sequence of a single purified protein as IVGGQEAPRSKW, which is identical to that reported for human mast cell trypsin (29). There were no other proteins detectable in the blot used for N-terminal sequencing. Preparations of trypsin used in this study had sp. act. between 2.5 and 3 μU/μg of protein. Endotoxin concentrations in the trypsin samples were less than 10 pg/μg of trypsin.
Cell mitogenesis

Tryptase stimulated thymidine incorporation in HUVEC in a dose-dependent manner at concentrations up to 20 mU/ml, and thereafter there was a decline at higher concentrations (Fig. 1A). The degree of stimulation at 20 mU/ml was similar to that achieved with the positive control ECGF. Preincubation of tryptase with the protease inhibitor leupeptin or heat inactivation of tryptase significantly reversed the increase in thymidine incorporation observed (Fig. 1B). Assessment of tryptase activity toward the chromogenic substrate BAPNA indicated inhibition of greater than 95% with both of these treatments. Addition of either leupeptin or heparin alone was without effect on thymidine incorporation. When cells were enumerated by direct cell counting following a 48-h incubation with either tryptase (20 mU/ml), medium alone, or ECGF (1.6 ng/ml), the cell numbers were: 3.7 ± 0.1 × 10^5, 3.8 ± 0.1 × 10^5, and 6.2 ± 0.1 × 10^5, respectively (mean ± SEM from three separate experiments). Application of the MTT cell proliferation assay also failed to reveal an increase in cell numbers in response to the incubation with tryptase. In fact, tryptase did induce some diminution in cell number following a 48-h incubation with tryptase, while FCS included as a positive control stimulated an increase of more than twofold in cell number (Fig. 2A). Viability, as assessed by the trypan blue exclusion technique, was approximately 90% in all cases, and there was no decrease with increasing concentrations of tryptase. When the same preparations of tryptase were added under similar conditions to the H292 epithelial cell line, cell proliferation was observed (Fig. 2B), consistent with a previous report with these cells (14).

Tryptase-induced cytokine release

Tryptase stimulated an increase in IL-8 release from HUVEC, which was apparent within 6 h of addition (Fig. 3A). There was a dose-dependent release of IL-8 from HUVEC over a range of tryptase concentrations (Fig. 3B). Variation in tryptase-induced HUVEC IL-8 release was observed from different donors. Preincubation of tryptase with the protease inhibitors leupeptin (20 μg/ml) or benzamidine (15 μg/ml) before adding to the cells significantly reduced IL-8 release (Fig. 4A), as did heat inactivation of tryptase and depletion of samples of tryptase by immunoprecipitation with the tryptase-specific mAb AA5 (Fig. 4B).
catalytic activity of tryptase toward BAPNA was reduced by more than 95% following each of these treatments. No significant alteration in IL-8 release from HUVEC was observed with the addition of leupeptin or benzamidine alone, or with heparin at the concentrations used to stabilize tryptase (Fig. 4, A and B). TNF-α was also effective in inducing increased IL-8 release, but with this stimulus, preincubation with either leupeptin (20 μg/ml) or benzamidine (15 μg/ml) had little effect on TNF-α-induced IL-8 release (Fig. 4C).

Using the same supernatants in which levels of IL-8 were determined, we investigated the presence of certain other endothelial derived cytokines. TNF-α (10 U/ml) stimulated the release of substantial quantities of GM-CSF from HUVEC (234 ± 48 pg/ml, n = 6), but levels were undetectable (<8 pg/ml) in the supernatants from cells incubated for 24 h with a range of concentrations of tryptase (5–100 mU/ml). Similarly, incubation of cells with a standard concentration of tryptase (50 mU/ml) for periods ranging from 1 to 48 h failed to provoke the release of detectable concentrations of GM-CSF. Neither the precursor nor the mature forms of IL-1β were detected (<15 pg/ml and <4 pg/ml, respectively) following incubation of endothelial cells with tryptase or with TNF-α at any of the concentrations or time points tested. All experiments were performed on six separate occasions.

Expression of mRNA

There was constitutive expression of HUVEC mRNA for IL-8 in untreated controls, but with the addition of tryptase or TNF-α, both induced an increase in expression (Fig. 5). Tryptase also stimulated an increase in mRNA expression for IL-1β (for which constitutive expression was not observed), but not to the same extent as was induced with TNF-α. Although TNF-α elicited expression of mRNA for GM-CSF in the same samples, this was not the case for tryptase. The APRT controls confirmed that there was equal loading of RNA.

Adhesion molecule expression

Incubation of HUVEC with tryptase at concentrations from 5 to 100 mU/ml had negligible effect on the expression of E-selectin, VCAM-1, or ICAM-1 at 4, 12, and 24 h, respectively, whereas TNF-α was able to stimulate marked increases in the expression of all three adhesion molecules (Table I). Similarly, a standard dose of tryptase (50 mU/ml) incubated with HUVEC for 1, 2, 4, 6, 24, or 48 h failed to alter the expression of any of the adhesion molecules studied (data not shown).

Discussion

Tryptase can stimulate profound alterations in the behavior of endothelial cells. Our findings indicate that this major mast cell product can induce endothelial cells to produce inflammatory cytokines. Although tryptase did not stimulate proliferation or alter the expression of adhesion molecules on HUVEC, the ability to induce the selective secretion of IL-8 and the expression of mRNA for IL-1β could be important for the recruitment of inflammatory cells to sites of mast cell activation.

of adding heparin alone. C, Effect of leupeptin or benzamidine on TNF-α (10 U/ml)-induced IL-8 release. T, tryptase (50 mU/ml); Leu, leupeptin (20 μg/ml); Benz, benzamidine (15 μg/ml); HT, heat-inactivated tryptase (formerly 50 mU/ml); IP, immunoprecipitated tryptase (formerly 50 mU/ml); Hep, heparin alone (5, 10, and 20 μg/ml). Results for A and B are expressed as the mean ± SEM of three separate experiments performed in triplicate, and those for C represent the mean ± SEM of triplicate determinations. *p < 0.05 compared with control values; †p < 0.05 compared with response with tryptase (50 mU/ml).
The tryptase used in this study was of high purity and activity, and as endothelial cells can respond to relatively small quantities of endotoxin, considerable care was taken to ensure that endotoxin concentrations in all samples were very low. The use of a polymyxin B column to remove endotoxin from the purified tryptase ensured that endotoxin represented less than 10 pg/μg of protein, a concentration that is substantially below those capable of stimulating cytokine release from endothelial cells (30–32). Furthermore, the inhibitory actions of protease inhibitors, of heat inactivating the enzyme, and immunoprecipitation experiments provide compelling evidence that tryptase was responsible for the effects observed.

Tryptase of both human and canine origin has been reported to be a growth factor for a number of cell types, including fibroblasts (12, 13), epithelial cells (14), and smooth muscle cells (15), in addition to dermal microvascular endothelial cells (16). We found that, although tryptase could stimulate an increase in thymidine incorporation in HUVEC, there were no associated cell proliferation responses, even at concentrations of tryptase substantially higher than those effective in studies with other cell types (12–16). In fact, a diminution in cell number was noted when the MTT cell proliferation assay was used. The observed diminution in cell number did not appear to be due to a direct cytotoxic action of tryptase, as assessed by trypan blue exclusion. Therefore, the decline in cell number observed would suggest that tryptase may actually arrest the growth of HUVEC. Consistent with a previous report (14), the same preparation of tryptase was nonetheless able to induce proliferation of the H292 epithelial cell line, indicating that the differences observed reflect variations in the responsiveness of different cell types.

The inability of tryptase to promote endothelial cell proliferation in these studies appears to be at variance with the findings of Blair et al. (16), who have reported recently that tryptase can stimulate endothelial cell tube formation. The apparent discrepancy may relate to the different sources of endothelial cells, which in our studies were derived from human umbilical veins, and in those of Blair and colleagues were from the microvasculature of foreskin tissue. Endothelial cells of different tissue origins have been found to respond differently to certain stimuli (33–36). Thus, for example, the serine protease thrombin can act as a growth factor for microvascular endothelial cells derived from lung, but not those from brain tissue (36). Alternatively, the use of serum-free conditions in our studies may have reduced the potential for tryptase to interact with growth factors in stimulating fibroblast proliferation (12), and endothelial cell tube formation. The apparent discrepancy may relate to the different sources of endothelial cells, which in our studies were derived from human umbilical veins, and in those of Blair and colleagues were from the microvasculature of foreskin tissue. Endothelial cells of different tissue origins have been found to respond differently to certain stimuli (33–36). Thus, for example, the serine protease thrombin can act as a growth factor for microvascular endothelial cells derived from lung, but not those from brain tissue (36). Alternatively, the use of serum-free conditions in our studies may have reduced the potential for tryptase to interact with growth factors in stimulating fibroblast proliferation (12), and there may be similar interactions with growth factors for endothelial cells. The possibility cannot be excluded also that the increase in thymidine incorporation may reflect interference in the processing of thymidine into the cell DNA, and it has been recognized previously that thymidine incorporation may not always provide an accurate indication of cell proliferation (37, 38). Increases in DNA synthesis in the absence of an increase in cell number have been reported in IL-1-stimulated HUVEC (39), as well as in hepatocytes (40). We would conclude that, in contrast to its effects on
various other cell types, tryptase by itself is not a growth factor for HUVEC.

Tryptase stimulated IL-8 release in a dose-dependent fashion from HUVEC. The ability of tryptase to stimulate IL-8 release from the H292 epithelial cell line has been noted previously with slightly lower concentrations (14), but this is the first report that tryptase can induce cytokine release from a primary cell culture. Moreover, by reverse-transcription PCR we have established that tryptase can elicit an increase in mRNA for IL-8, suggesting the ability to stimulate de novo synthesis of IL-8. Tryptase was more selective in its actions on endothelial cells than was TNF-α, and did not stimulate a concomitant increase in either mRNA or protein for GM-CSF, or alter adhesion molecule expression. IL-8 is well established as a potent chemotaxiant and an activator of neutrophils (41–43), as well as of eosinophils (44), and leukocyte migration may be mediated in part by IL-8 expression on the endothelial cell surface, in addition to its release from the cell (45). The ability of TNF-α to stimulate endothelial IL-8 release has been proposed as a key process in granulocyte recruitment at sites of mast cell activation (23). Although tryptase is not as potent a stimulus of IL-8 release as TNF-α, it is likely to be released in much greater quantities, and may therefore play an important role in initiating mast cell-induced inflammation. This would be in keeping with studies with guinea pigs and mice in which the injection of human tryptase has been found to induce the accumulation of neutrophils and eosinophils in vivo (5).

Although tryptase, like TNF-α, induced the expression of mRNA for IL-1β in HUVEC, we were unable to detect IL-1β either in its precursor or mature form in supernatants following incubation with tryptase or TNF-α for up to 48 h. It has been reported that TNF-α has a negligible effect on endothelial IL-1β release (46–49), even though it induces an accumulation of IL-1β mRNA (32, 48). These reports, together with our own findings with tryptase and TNF-α, suggest that endothelial cell IL-1β production is a closely regulated process. It is possible that tryptase and TNF-α prime endothelial cells in preparation for another stimulus that could then induce IL-1β release. Alternatively, endothelial cells may require simultaneous activation by several stimuli before IL-1β is released, as has been indicated in studies with fibroblasts (50). At sites of mast cell activation, endothelial cells will be exposed not just to tryptase, but also to a range of other products of mast cells and other cells types, and together with other factors, it could contribute to endothelial cell IL-1β production. Our findings do suggest that tryptase either alone or in combination with other mediators can have an important role in endothelial cell cytokine production.

The actions of tryptase on HUVEC appear dependent on an intact catalytic site, as they were significantly inhibited either by preincubation with the protease inhibitors leupeptin or benzamidine, or by heat inactivation of the enzyme. These treatments were able to inhibit more than 95% of the enzymatic activity toward the chromogenic substrate, but appeared rather less effective in reducing the increases in thymidine incorporation and IL-8 release. The apparent ability of inactive tryptase to retain activity as a growth factor has been noted previously with cultures of fibroblasts (12) and smooth muscle cells (15), and it is possible that tryptase may, as described for thrombin (51), modulate HUVEC behavior by mechanisms that are both dependent and independent of the catalytic site. However, in the present studies, the responses to inactivated tryptase did not differ significantly from those with buffer alone, and the trends observed could relate simply to the residual tryptase activity in the preparations.

The nature of the initial proteolytic event is not clear. While cleavage of an exogenous substrate by tryptase cannot be excluded, the maintenance of these cells in serum-free conditions makes this less likely to have occurred. Tryptase may interact directly with the cell surface, possibly, like thrombin, by cleaving a protease-activated receptor (PAR). The thrombin receptor (PAR-1) is expressed on HUVEC, but appears not to be activated by tryptase (52). Moreover, the actions on HUVEC of thrombin and a peptide agonist of PAR-1 (representing the new N-terminal region of the cleaved receptor) differ from those of tryptase and are able to up-regulate expression of E-selectin, as well as inducing IL-8 secretion (25). PAR-2 is also expressed on HUVEC, and a recent report has suggested that tryptase can activate this receptor, albeit less effectively than trypsin (52). However, activation of PAR-2 with a peptide agonist has been found to result in a strong proliferative response in HUVEC (53), an effect that was not observed with tryptase in the present study. The activation of PAR-2 by tryptase would seem, therefore, to be relatively weak, and this alone is unlikely to account for the actions of this protease on endothelial cells, which we have observed. The presence of PAR other than PAR-1 and PAR-2 has been suggested by the observation that a PAR-1/PAR-2 agonist can still initiate a response in HUVEC, even when the cells have been desensitized to PAR-1 and PAR-2 (54). Recently, the characterization of a PAR-3 on HUVEC has been described, a receptor that can be cleaved by thrombin (55).

Further studies are required to characterize the family of PAR on endothelial cells, and to determine the extent to which the actions of tryptase on this cell type may involve their activation. The ability of tryptase to alter endothelial cell behavior may be important at sites of mast cell activation. In particular, the selective release of IL-8 from endothelial cells in response to tryptase could provide a signal for the accumulation of inflammatory cells.

Acknowledgments

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References


