The Mitogenic Effect of Thrombin in Vascular Smooth Muscle Cells Is Largely Due to Basic Fibroblast Growth Factor*

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Mitogenesis induced by most polypeptide growth factors is mediated by either G-protein- or tyrosine kinase-linked receptor pathways. Thrombin, a potent mitogen for vascular smooth muscle cells, activates a G-protein-coupled receptor but also requires tyrosine kinase activity for its mitogenic effect (Weiss, R. H., and Nucifetti, R. (1992) J. Biol. Chem. 267, 5608–5613). Since this growth factor elicits a synergistic effect on DNA synthesis when applied to cells concurrently with basic fibroblast growth factor (bFGF), we suspected that the two growth factors have points of convergence in their signaling pathways. We now show that when 1 unit/ml thrombin is removed after an incubation period of from 4 h to 15 min prior to 15 ng/ml bFGF addition, its synergistic effect with bFGF on mitogenesis in vascular smooth muscle cells is preserved. Furthermore, appearance of bFGF in the cellular lysate is maximal 1 h after the addition of 1 unit/ml thrombin. While monoclonal antibody to bFGF inhibits thrombin’s mitogenic effect by 63% at 30 μg/ml, it lacks an inhibitory effect on platelet-derived growth factor-BB-induced mitogenesis. The inhibitory effect of bFGF antibody on thrombin’s growth is completely reversed by the addition of bFGF. These data demonstrate that the presence of bFGF is essential for thrombin to exert its full mitogenic effect in vascular smooth muscle cells, providing an example of a system where a tyrosine kinase-linked growth factor receptor system can act as an essential intermediary in the mitogenic signaling pathway of a G-protein-coupled receptor.

Polypeptide growth factors transmit their mitogenic signals into eukaryotic cells through two predominant receptor pathways (Refs. 1–3 and references therein). The receptors for epidermal growth factor, PDGF, and FGF activate cellular tyrosine kinases (2–4), whereas bombesin, vasopressin, serotonin, and thrombin employ G-protein-coupled receptors for their mitogenic signaling (3). Upon association with their respective receptors, growth factors trigger a variety of biochemical events leading to DNA replication and cell division (2, 5). While these two receptor types have been considered to activate distinct signaling pathways, evidence is now accumulating that they interact at multiple levels (3, 6). Thrombin, a protease which stimulates growth via a proteolytic mechanism employing a tethered ligand (7), is associated with a receptor which falls into the 7-transmembrane spanning class of receptors typical of those coupled to G-proteins. This growth factor stimulates DNA synthesis to a magnitude unusual for the G-protein-coupled receptors (8), suggesting that other mitogenic signaling pathways are involved.

We have previously demonstrated that thrombin requires tyrosine kinase activity for its mitogenic action, but not for its effect on intracellular free calcium release in neonatal rat VSM cells (6). In addition, the synergistic effect of thrombin and bFGF on both mitogenesis (6) and inositol phosphate metabolism (3) suggests that these two growth factors possess points of convergence at some level in their signaling pathways. Work in other laboratories has established that inositol phosphate hydrolysis alone is not sufficient for the mitogenic effect of thrombin (8), and that the inositol phosphate hydrolysis induced by tyrosine kinase-linked growth factors is not essential for their growth-promoting action (9). We therefore suspected that the intersection of the thrombin and bFGF signaling pathways was at the level of the receptor-coupled tyrosine kinase, with the bFGF receptor supplying the tyrosine kinase involved in thrombin’s mitogenic signaling pathway. We now show that bFGF is a requisite intermediary for the full mitogenic action of thrombin, but not of PDGF, in vascular smooth muscle cells.

EXPERIMENTAL PROCEDURES

Materials—Highly purified α-thrombin was generously supplied by John W. Fenton II (New York State Department of Health, Albany, NY). Human recombinant bFGF, human recombinant PDGF-BB homodimer, monoclonal mouse anti-bovine bFGF, and monoclonal mouse anti-human PDGF-BB antibodies were obtained from UBI (Lake Placid, NY). Goat anti-mouse horseradish peroxidase-linked IgG was obtained from Bio-Rad. Reagents for the Enhanced Chemiluminescence system and [3H]thymidine were obtained from Amer sham. All other reagents were obtained from Sigma.

Cell Culture—Primary cultures of VSM cells from newborn rat were established by Peter Jones, University of Southern California (10). From these primary cultures, the R22D clone was established and generously supplied to us at passage 15. Cells from passages 15–24 were used for the present studies. The cells were maintained in minimal essential medium with 10% fetal bovine serum, 2% tryptose phosphate broth, penicillin (50 units/ml), and streptomycin (50 units/ml) in a humidified atmosphere of 5% CO2, 95% air at 37°C. Culture medium was changed every other day until the cells were confluent. The cells were growth-arrested by placing them in quiescence medium containing minimal essential medium, 20 mM HEPES (pH 7.4), 5 μg/ml transferrin, 0.5 mg/ml bovine serum albumin, penicillin (50 units/ml), and streptomycin (50 units/ml). Quiescence medium was changed daily for 2 days prior to the experiment. Cells were harvested with 5 ml of trypsin and subcultured at a 1:10 dilution weekly.

DNA Synthesis—Cultured VSM cells were harvested, plated onto 24-well plates, and grown to confluence. The confluent monolayers were then placed in quiescence medium for an additional 2 days. Where indicated, freshly thawed antibody was added 2 days following

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The abbreviations used are: PDGF, platelet-derived growth factor; bFGF, basic fibroblast growth factor; VSM, vascular smooth muscle.
exposed to growth factors as indicated. After 24 h, cells were pulsed with [3H]thymidine. The cells were then fixed with 1 ml of 15% trichloroacetic acid, and trichloroacetic acid-insoluble radioactivity was measured in a scintillation counter.

**Western Blots**—VSM cells were grown to confluence on 6- or 10-cm dishes and quiesced as above. Thrombin (1 unit/ml) was added to the medium for the indicated times. The monolayer of cells was washed twice with ice-cold phosphate-buffered saline and lysed in place by the addition of ice-cold RIPA buffer (10 mM Tris base, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 100 μM sodium orthovanadate, 2 mM iodoacetic acid, 1 mM phenylmethylsulfonyl fluoride (freshly prepared), 0.1% bovine serum albumin, 0.5% Nonidet P-40, and 5 μM ZnCl2 (pH 7.1 (1 ml/60-mm dish)). After 30 min of incubation at 4 °C, the lysate was scraped off with a rubber spatula and centrifuged at 30,000 rpm at 4 °C for 30 min. The supernatant was saved and the protein concentration determined spectrophotometrically at A280 with the Bio-Rad protein concentration reagent. Aliquots containing identical amounts of protein for each lysate were boiled in a boiling water bath for 5 min, loaded onto a 4%–15% polyacrylamide gel with stacking gel with appropriate molecular weight standards, and electrophoresed at 25 mA for 2–4 h. Subsequently, the gel was electrophoretically transferred onto a nitrocellulose filter in transfer buffer (192 mM glycine, 25 mM Tris, pH 7.5, 0.1% Tween 20), washed in TBS-T, and incubated at room temperature for 1 h in a 1:2000 dilution of monoclonal anti-bFGF. The filter was washed again in TBS-T and incubated for 1 h with a 1:10,000 dilution of goat anti-mouse horseradish peroxidase-coupled IgG. The final washes were with TBS-T. The Enhanced Chemiluminescence system was used for detection. Enhanced Chemiluminescence reagents were mixed 1:1 and applied to the filter which was subsequently exposed to film for from 15 s to 15 min and the film processed.

**RESULTS**

The Synergistic Effect of Thrombin and bFGF Is Not Dependent on Their Simultaneous Presence in the Medium—To distinguish whether the synergistic effect of thrombin on bFGF-stimulated mitogenesis was due to the direct and simultaneous action of these growth factors on a substrate, or by means of thrombin-induced production of an intermediary, the temporal nature of the interaction between these two growth factors was investigated. Thrombin was added to the growth medium at various times before the addition of bFGF, and the cells were washed thoroughly to remove residual thrombin before the latter's addition. In the experiment shown in the left half of Fig. 1, both bFGF and thrombin were added simultaneously to VSM cells. bFGF increased DNA synthesis by 3 times basal (column F), and thrombin increased DNA synthesis by 6.5 times basal (column T). Thrombin and bFGF added together increased DNA synthesis by 14.1 times basal, displaying a marked synergistic effect of these two growth factors (column T + F), as previously described (6). In the experiment shown in the right half of Fig. 1, thrombin was washed off of the cells before the addition of bFGF. When thrombin was washed off after a 4-h incubation, the effect of bFGF was to increase mitogenesis 10.5 times basal (column −4). Although slightly diminished compared to the wells which had not been washed, the synergistic effect was preserved despite the fact that thrombin and bFGF were not present simultaneously. Similar results were obtained with cells from which thrombin had been removed 2 h (column −2) and 15 min after its addition (column −0.25). This outcome was not due to effects of residual thrombin left on the plate or bound to receptor, since when thrombin was washed off after an instantaneous incubation (column 0), the synergistic effect was abolished. Therefore, since thrombin does not have to be present in the media for its synergistic effect with bFGF to occur, its interaction with the bFGF system is likely being mediated through the production of a substance or alteration of a substrate removed in time from the application of thrombin.

**Thrombin Causes Appearance of bFGF in Cell Lysate**—bFGF has been shown to be transcribed in capillary endothelial cells in response to thrombin (11), and it has been shown to be an essential intermediary in the effect of PDGF on VSM migration (12) and on cellular repair after vascular injury (13). Therefore, one explanation for the synergistic effect of thrombin and bFGF (3, 6) was that thrombin was causing the cell to produce bFGF, and that the latter substance was acting as the intermediary described above. Synergy would occur under these circumstances because the bFGF receptor would "see" agonist in excess of that added exogenously. Furthermore, it is possible that bFGF produced in this manner accounts for the tyrosine kinase required for thrombin-induced mitogenesis and which was inhibited by herbimycin A (6). To demonstrate that bFGF is produced by these cells in response to thrombin, we examined appearance in cell lysates of immuno-reactive bFGF. Lysates obtained from VSM cells which had been stimulated by thrombin for various times was subjected to Western blotting and probed with monoclonal anti-bFGF antibody (14). bFGF, initially undetectable in the lysate, appears 1 h after thrombin addition (Fig. 2a, arrow) and is no longer present at 2 h. To confirm the identity of the 17.5-kDa band as bFGF, an authentic bFGF standard was also electrophoresed as a positive control (Fig. 2a, lane F). To further establish specificity of the antibody for bFGF, the filter was reprobed in the presence of 30 ng/ml bFGF in the first antibody solution in an attempt to inhibit specific binding of the antibody to bFGF on the filter (Fig. 2b). The 17.5-kDa band present in both the 1-h time point and in the bFGF standard did not appear in the reprobed filter (Fig. 2b, arrow), verifying that the antibody did in fact recognize bFGF.

**bFGF Is Essential for the Full Mitogenic Action of Thrombin but Not of PDGF-BB**—To demonstrate that production of
bFGF is involved in thrombin's mitogenic action, we utilized monoclonal antibodies against bFGF to specifically neutralize its biologic effect. If extracellular bFGF is required for the mitogenic effect of thrombin, incubation of VSM cells with bFGF antibody would be expected to precipitate this growth factor and render it unable to bind its receptor (12–14). When added to the serum-free medium-bathing quiescent cells immediately prior to the addition of thrombin, this antibody demonstrated dose-dependent inhibition of thrombin-induced DNA synthesis by from 22% at 0.3 μg/ml to 63% at 30 μg/ml (Fig. 3a, squares). To confirm that this effect was specific to bFGF antibody and was not a general phenomenon involving all tyrosine kinase-coupled receptor agonist antibodies, we studied the effect of monoclonal antibody to PDGF-BB in this system. Similarly to FGF, the PDGF receptors belong to the tyrosine kinase-coupled family of growth factor receptors (2, 4). Consistent with our observation that PDGF-BB and thrombin do not display mitogenic synergy,2 we observed no significant inhibitory effect of this antibody on thrombin-induced DNA synthesis when added at similar concentrations to those used for bFGF antibody (Fig. 3a, circles). In fact, there was a minor stimulatory effect of this antibody on DNA synthesis. To verify that the inhibition of thrombin's mitogenic action was not due to a toxic effect of either bFGF antibody or of IgG, we examined the effect of adding bFGF antibody in the absence of thrombin to quiescent cells; there was no effect on basal DNA synthesis in VSM cells of this antibody added alone (Fig. 3a, triangles).

To further investigate specificity of the inhibitory effect of bFGF antibody to thrombin's mitogenic effect, we added PDGF-BB to VSM cells in the presence or absence of bFGF antibody. PDGF-BB increased DNA synthesis 11-fold over control. In contrast to the results obtained with thrombin, bFGF antibody, when added to the media at a concentration which decreased thrombin-induced DNA synthesis by 50% (20 μg/ml; see Fig. 3a) and incorporation of [3H]thymidine was assessed as described (8). Each data point represents the mean ± S.E. of three wells and is representative of one of two to five separate experiments.

The Inhibitory Effect of bFGF Antibody on Thrombin-induced Mitogenesis Is Reversed by bFGF—To illustrate that bFGF antibody specifically inhibits a bFGF-mediated process, we next asked if the inhibitory effect of this antibody could be reversed by the addition of bFGF. When 30 ng/ml bFGF was added to thrombin-stimulated cells at the same time as the bFGF antibody (20 μg/ml), the inhibitory effect of the antibody was abolished (Fig. 4). Furthermore, bFGF at

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2 R. H. Weiss, unpublished observations.
Effect of bFGF Antibody on Thrombin-induced Mitogenesis

thrombin's mitogenic activity (see Fig. 1). Immediately before thrombin addition (7.1 unit/ml), freshly thawed mouse monoclonal antibody to bovine bFGF (αF, 20 μg/ml) or human bFGF (F, 30 ng/ml) was added to specified wells. 18 h later, all cells were pulsed with [³H]thymidine and incorporation of [³H]thymidine was assessed as described (8). Each data point represents the mean ± S.E. of three wells and is representative of one of two separate experiments.

this concentration increased DNA synthesis 75% greater than thrombin alone, reminiscent of the synergistic effect of thrombin and bFGF on VSM cell mitogenesis (see Fig. 1).

DISCUSSION

While protein tyrosine kinase activity is essential for the mitogenic action of thrombin in VSM cells (6), the role of tyrosine kinases in the signaling pathway of G-protein-coupled growth factor receptors in general has not been elucidated. Since the mitogenic activity of thrombin is unusually potent for a receptor of the G-protein type (8), and because thrombin and bFGF cause synergistic activities on inositol phosphate hydrolysis (5) and DNA synthesis (6), we suspected that these pathways had points of intersection in the signal transduction cascade of at least some polypeptide growth factors. We now demonstrate an essential role for the tyrosine kinase-coupled receptor for bFGF in thrombin's mitogenic pathway by showing that (i) thrombin need not be present to display its synergistic effect with bFGF, suggesting production of a substance removed in time from its addition, (ii) bFGF appears in cellular lysate after the addition of thrombin, and (iii) specific antibody-inhibitable action of bFGF is essential for the full mitogenic effect of thrombin on VSM cells.

Since antibodies added to growth media do not act intracellularly, the bFGF antibody exerts its inhibitory effect on extracellular bFGF. However, as bFGF does not possess a consensus signal peptide, it has not been evident whether this growth factor is secreted in order to act extracellularly in living cells (15). Several authors have shown that bFGF is released under certain circumstances leading to autocrine or paracrine activities (16–18), but whether the bFGF neutralized by antibody in our studies is due to secreted protein or to that released from lysed dead cells (19) is an issue which we have not addressed. It is unlikely, however, that bFGF released by dead cells could account for as much as 60% of thrombin's mitogenic activity (see Fig. 3).

While the specific tyrosine kinase inhibitor herbimycin A diminishes thrombin's mitogenic effect by greater than 90% (6), we observed only a 60% inhibition by bFGF antibody. This finding may be due to (i) incomplete immunoneutralization of extracellular bFGF by addition of exogenous antibody at the concentration used, (ii) the presence of an intracellular agonist-receptor pathway for bFGF which is unreacheable by bFGF antibody, as has been suggested for other growth factors (20), or (iii) the involvement of a bFGF-independent tyrosine kinase pathway inhibitable by herbimycin A in thrombin's signaling cascade.

That a tyrosine kinase is activated by thrombin and plays an essential role in its mitogenic signaling cascade is not surprising in light of the magnitude of growth stimulation by this growth factor, unusual for the G-protein-coupled receptor agonists (8). Receptors with tyrosine kinase activity have been demonstrated to have parallel roles as intermediaries in other signaling events: PDGF-A chain production is required for the effect of interleukin-1 on cell migration (21), and bFGF is necessary for the effect of PDGF on vascular smooth muscle cell migration (12) and for proliferation of smooth muscle cells after vascular injury (13). Pouyssegur and associates (22), observing that synthetic thrombin receptor "tethered ligand" peptides stimulated phospholipase C activity but had no effect on mitogenesis, postulated the existence of a thrombin receptor signaling pathway likely to involve tyrosine kinase activation (22). Work in our laboratory has similarly shown that thrombin's phospholipase C stimulatory activity can be separated from its mitogenic action (6). However, this report is the first to our knowledge to demonstrate that a tyrosine kinase-linked growth factor receptor complex is acting as a requisite intermediary in the mitogenic signaling pathway of a receptor coupled to a G-protein. Further study is needed to clarify the interaction between these two classes of growth factor and to determine if a general phenomenon of the G-protein-coupled receptor mitogens is the requirement for activation of a tyrosine kinase.

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