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The Role of p38 MAP Kinase in TGF-β1-Induced Signal Transduction in Human Neutrophils[1]

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Transforming growth factor-β1 (TGF-β1) is the strongest chemoattractant yet described for human neutrophils. It activates neither phospholipase C nor phospholipase D. It does not induce rises in intracellular calcium, degranulation, or superoxide production. The signaling pathways utilized by TGF-β1 are largely unknown. This report demonstrates that TGF-β1 activates p38 MAP kinase. The kinase inhibitor SB203580 blocks the chemotactic responses as well as actin polymerization induced by TGF-β1. Potential cellular targets of the p38 MAP kinase pathway which could mediate these function are discussed.

Key Words: **TGF-β1; p38 MAP kinase; neutrophil; chemotaxis.**

Recently, a novel signaling cascade that is analogous to the growth factor induced mitogen activated protein kinase cascade has been described (1). This signaling cascade is activated through cellular stresses, inflammatory cytokines, and chemotactic factors (1-3). Activation of this protein kinase cascade causes the phosphorylation and activation of a protein kinase termed p38 MAP kinase (4). One of the targets of activated p38 MAP kinase is MAPKAP kinase 2. Phosphorylated and activated MAPKAP kinase 2 has been used as a specific intracellular marker to indicate an activated p38 MAP kinase pathway (1). The major substrates for MAPKAP kinase 2 include HSP27 (6), myosin light chain (7), LSP1 (8), tyrosine hydroxylase (9), and cAMP regulatory element binding protein (10). SB203580, which is a specific inhibitor of this pathway, binds to and inactivates p38 MAP kinase (11, 12).

Transforming growth factor-β1 (TGF-β1) is a very potent chemoattractant for human neutrophils (13). Unlike other chemotactic factors such as N-formyl-Met-Leu-Phe (FMLP), TGF-β1 does not stimulate neutrophil degranulation or superoxide production (13). It also does not increase the intracellular level of calcium or diacylglycerol (DAG) (13, 14). The signaling cascade induced by TGF-β1 remains unknown. Recently, neutrophil p38 MAP kinase has been suggested to be involved in the chemotactic response induced by FMLP (2,3). In this report, we investigate the possibility that p38 MAP kinase is involved in mediating the chemotactic signals generated by TGF-β1. We observed that although TGF-β1 stimulated weakly the p38 MAP kinase pathway, activation of p38 MAP kinase appears to be required for chemotaxis and actin-polymerization induced by TGF-β1.

MATERIALS AND METHODS

Materials. Recombinant TGFβ1 was purchased from R & D systems (Minneapolis, MN). FMLP was purchased from Sigma chemical company (St. Louis, MO). Stock solutions of FMLP were prepared by diluting the FMLP to 5×10^{-2} M in DMSO and storing at -70°C. Human peripheral blood neutrophils were obtained from healthy volunteer donors as previously reported (15). The neutrophils were washed, counted and resuspended in Hanks balanced salt solution (HBSS, Gibco BRL, Grand Isle, NY) before use.

Chemotaxis. Human neutrophils were collected and distributed to the upper chambers of the wells of a Boyden apparatus at a density of 2×10^6 per well. Upper and lower chambers were separated with 5 μM membranes (Millipore, Bedford, MA). The cells were incubated for 2 hr with or without 1 μM SB203580 present in the upper chambers and with chemoattractant in the lower chambers of the wells. The membranes were then stained as described (16). Using an Olympus BX40 microscope (Olympus America, Lake Success, NY) the number of neutrophils in a single 10x field of the stained membrane was determined. The focus rotor was then decreased by 10 units, and the number of cells at that level determined. This process was repeated so that cell numbers at each of 4 levels of the same membrane location could be determined.

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Abbreviations: TGF-β1, transforming growth factor-β1; MAP kinase, mitogen activated protein kinase; MAPKAP kinase 2, MAP kinase activated protein kinase 2; FMLP, N-formyl-met-leu-phe; SDS/PAGE, Sodium dodecyl sulfate/polyacrylamide gel electrophoresis; DAG, diacylglycerol; PLC, phospholipase C; HBSS, Hanks balanced salt solution; PBS, phosphate buffered saline.

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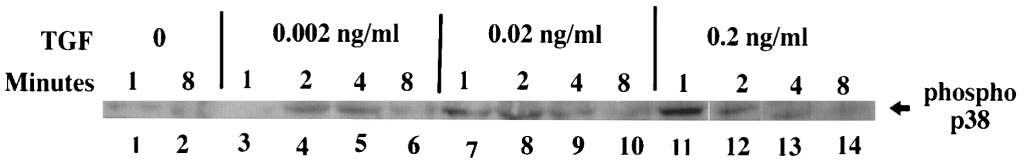

FIG. 1. p38 MAP kinase activation induced by TGF-β1. Neutrophils (4×10^7 cells/ml) were stimulated with 0 (lanes 1 and 2), 0.002 ng/ml (lanes 3-6), 0.02 ng/ml (lanes 7-10), or 0.2 ng/ml (lanes 11-14) of TGF-β1 at 37°C for indicated times. The reactions were stopped by the addition of SDS stopping solution (2). Samples were then analyzed by 12% SDS/PAGE and immunoblotted with anti-phospho p38 MAP kinase antibodies as described in the text.

Flow cytometric analysis of actin polymerization. Neutrophils were incubated with or without SB203580 (1 μM) for 20 minutes at 37°C. The cells were stimulated with 10^{-7} M FMLP or 0.2 ng/ml TGF-β1 for 3 min in buffer lacking Ca^{++}. The cells were then fixed with an equal volume of 4% paraformaldehyde. Fixation was done at 37° C for 10 min followed by an additional 20 min at room temperature. Cells were centrifuged (2 min. at 14,000 g) and resuspended in staining solution (1:250 dilution of stock NBD-phallacidin (Molecular Probes, Eugene OR) in 20 mg/ml lysolecithin/phosphate buffered saline (PBS)). The cells were then incubated at room temperature in the dark for 30 min. Finally, unbound phallacidin was removed by 3 washes in PBS/lysolecithin and samples were read on a Becton Dickinson FACScan cytometer with Cellquest analysis software. Analysis was performed using a linear scale fluorescence channel (FL1).

Determination of activated p38 MAP kinase. To detect activated p38 MAP kinase, purified neutrophils were suspended in HBSS to 4×10^7 cells/ml. The cells were then incubated at 37°C for 10 min, and stimulated with the indicated amount of TGF-β1 or buffer as a negative control. The cells were then incubated for 1, 2, 4, or 8 min at which time reactions were stopped with the addition of an equal volume SDS stopping solution (9% SDS, 1% β-mercaptoethanol, 15% glycerol, Bromophenol Blue). Samples were then boiled 5 min and resolved in 12% SDS/PAGE. Proteins within the gel were then transferred to nitrocellulose membranes and used in Western blots for the detection of phosphorylated p38 MAP kinase using polyclonal rabbit anti-phospho p38 antibody (New England Biolabs, Beverly, MA) (2).

Preparation and use of antibodies to phospho-MAPKAP kinase 2. MAPKAP kinase 2 is a specific target for p38 MAP kinase *in vivo* (1). The amino acid residue threonine 334 of MAPKAP kinase 2 has previously been shown as one of the major sites of phosphorylation recognized by p38 MAP kinase (7). Antisera specific for phosphothreonine 334 of MAPKAP kinase 2 was generated by Chiron Technologies (Clayton Victoria, Australia) in New Zealand white rabbits. The rabbits were immunized with the phosphopeptide KVPQT(PO_4)PLH conjugated to diphtheria toxoid. The amino acid sequence of this phosphopeptide corresponds to amino acids 330 to 337 of MAPKAP kinase 2. Antibodies were purified from the antisera by affinity chromatography.

Cells (2×10^7 cells/ml in HBSS containing 1 mM diisopropyl fluorophosphate (Sigma) and 1 mM $CaCl_2$) were stimulated with FMLP (2×10^{-8} M, positive control) or TGF-β1(0.2 ng/ml or 2 ng/ml) in order to detect the phosphorylation of MAPKAP kinase 2 in human neutrophils. When listed, samples were pre-incubated with 1 μM SB203580 for 20 min at 37°C. At indicated time points reactions were stopped by the addition of SDS stopping solution. Samples were then boiled for 5 min and separated on 10% SDS/PAGE and immunoblotted with the anti phospho-MAPKAP kinase 2 antibodies.

RESULTS

Activation of neutrophil p38 MAP kinase by TGF-β1. Treatment of human neutrophils with TGF-β1 induced the phosphorylation of p38 MAP kinase (Fig. 1). TGF-β1 (0.2 ng/ml) induced activation which showed transient kinetics peaking at 1 min and rapidly declining to basal levels (Fig. 1 lanes 11-14). A dose dependent increase in the level of phospho-p38 MAP kinase was noted when cells were treated with increasing concentrations of TGF-β1 (0.002 ng/ml to 0.2 ng/ml (Fig. 1, lanes 3-14)).

MAPKAP kinase 2 is a specific substrate of p38 MAP kinase within intact cells (7). We have prepared antibodies to phospho MAPKAP kinase 2 and used them to detect the activation of p38 kinase by immunoblotting. Stimulation of neutrophils with FMLP induced a transient increase of phosphorylated MAPKAP kinase 2 (Fig. 2, lanes 2-4). Two isoforms of MAPKAP kinase 2 (50 kD and 40 kD) were detected (17). TGF-β1 (0.2 ng/ml) treatment resulted in transient phosphorylation of MAPKAP kinase 2 (Fig. 2, lanes 8-10). The maximum effect induced by TGF-β1(2 ng/ml at 1 min, Fig. 2, lane 8) is approximately 42% of that induced by FMLP (10^{-7} M at 1 min, Fig. 2, lane 2). The activation of p38 MAP kinase induced by TGF-β1 can also be detected using ATF2 as an *in vitro* substrate in cell lysates as described (2) (data not shown). The phosphorylation of MAPKAP kinase 2 is inhibited by SB203580 (Fig. 2, lanes 11-13).

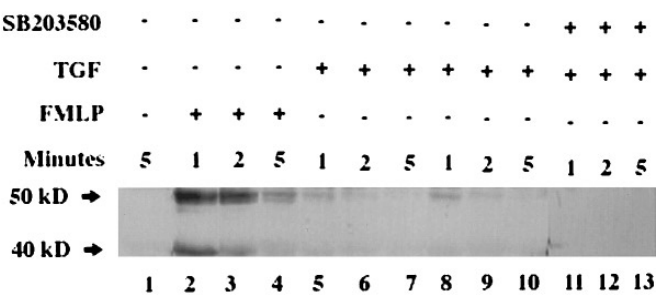

FIG. 2. Detection of phosphorylated MAPKAP kinase 2 induced by TGF-β1. Neutrophils were pretreated with (lanes 11-13) or without (lanes 1-10) SB203580 (1 μM) and then stimulated with TGF-β1 (0.2 ng/ml (lanes 5-7) 2 ng/ml (lanes 8-13)), FMLP (2×10^{-8} M, lanes 2-4) or buffer (lane 1) for various times as indicated. Reactions were stopped with SDS stopping buffer. Samples were analyzed by SDS/PAGE and immunoblotting with antibodies to phospho MAPKAP kinase 2 as described in the text. Two isoforms of MAPKAP kinase 2 of 40 kD and 50 kD were detected as shown by the arrows.

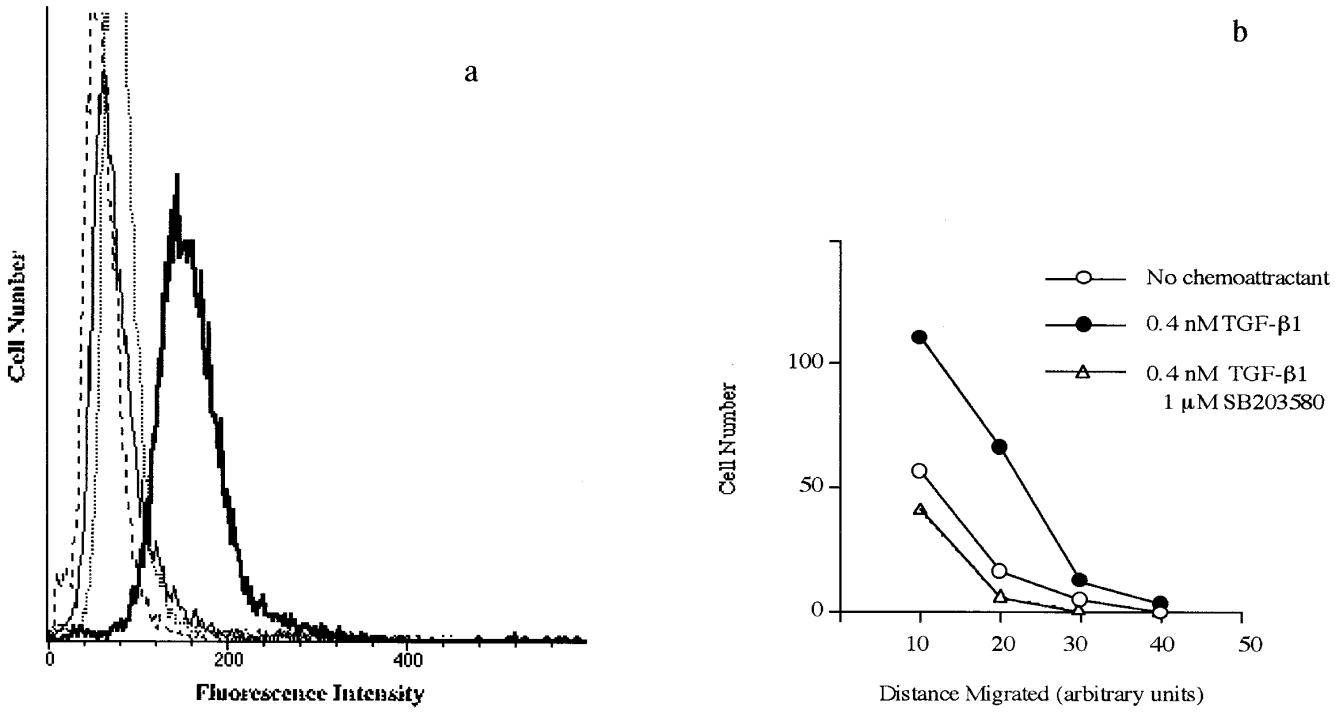

FIG. 3. SB203580 inhibits TGF-β1 induced actin polymerization (a) and chemotaxis (b). (a) Neutrophils (1×10^7 cells/ml) were pretreated with (· · ·, -·-) or without (–, —) SB203580 (1 μM) and then were stimulated with TGF-β1 (—, ---, 0.2 ng/ml) The cells were then fixed and stained for f-actin and f-actin was quantitated as described in the text. A representative experiment of 3 performed is presented. (b) Boyden chambers were used to measure chemotaxis of neutrophils toward TGF-β1. Neutrophils were incubated with (△) or without (○) SB203580 (1 μM) in upper chambers. They were then assayed for chemotaxis in the absence (○) or presence (●, △) of TGF-β1 (0.4 nM) in the lower chamber for 2 hrs as described in the text.

Actin polymerization and chemotaxis induced by TGF-β1. A rise in f-actin, as detected by NBD-phallacidin staining, was observed when neutrophils were stimulated with TGF-β1 (0.2 ng/ml) for 3 minutes. Pretreatment of the cells with the kinase inhibitor SB203580 (1 μM, 20 min) prevented the rise in f-actin (Fig. 3a). The level of f-actin induced by TGF-β1 reached maximum level in 3 min and then decayed in a manner similar to what has been described in FMLP induced actin polymerization (2, 3) (data not shown). SB203580 also reduced the ability of these cells to migrate in response to TGF-β1 (Fig. 3b).

DISCUSSION

Stimulation of p38 MAP kinase induced by FMLP has been reported in human neutrophils (2-4). The kinase inhibitor SB203580 has been used to demonstrate a role for this pathway in chemotaxis, superoxide generation, gene expression and adhesion in neutrophils (2-4). In this report, we demonstrate that TGF-β1 signaling also activates the p38 MAP kinase cascade. Specifically, stimulation of human neutrophils with TGF-β1 induced the phosphorylation of p38 MAP kinase and its *in vivo* substrate MAPKAP kinase 2. SB203580 inhibited the phosphorylation of MAPKAP kinase 2, f-actin polymerization and chemotaxis induced by TGF-β1. These results suggest the involvement of p38 MAP kinase in TGF-β1-induced actin-polymerization and chemotaxis.

Despite being a very potent chemoattractant, TGF-β1 is weaker than FMLP in stimulating the phosphorylation of p38 MAP kinase (data not shown) and the phosphorylation of MAPKAP kinase 2 (Fig. 2). This could be due to its inability to increase the levels of intracellular calcium and DAG. Full activation of both p38 MAP kinase and MAPKAP kinase 2 induced by FMLP require an intracellular calcium rise (19). Based on the previous findings with TGF-β1, high levels of intracellular calcium and DAG appear not to be required for chemotaxis (13). Neutrophils prepared from phospholipase C (PLC) β2 knock-out mice have increased chemotactic responses (20). These neutrophils lack intracellular calcium rise when stimulated by chemotactic factors. It is proposed that intracellular calcium rises may be inhibitory for chemotaxis (20). However, calcium regulated calcineurin has been reported to be important for neutrophil chemotaxis (21).

The inhibitory effect of SB203580 on f-actin polymerization and chemotaxis induced by TGF-β1 suggests

that cytoskeletal proteins may be important targets for p38 MAP kinase pathway. Cytoskeletal components that are targets of p38 MAP kinase are not known. With respect to MAPKAP kinase 2, several substrates which may regulate cytoskeletal structure are known. These substrates include myosin light chain (7), HSP27(6), and LSP1(8). Further work is needed to identify the molecular components involved in the chemotactic response regulated by the p38 MAP kinase pathway.

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