Soluble E-selectin Induces Monocyte Chemotaxis through Src Family Tyrosine Kinases*

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Cellular adhesion molecules such as E-selectin function to recruit leukocytes into the inflammatory lesions of diseases such as rheumatoid arthritis (RA) and atherosclerosis. Monocytes are the key components of the cellular infiltrates present in these disorders. We hypothesized that soluble E-selectin (sE-selectin) might mediate the chemotaxis of monocytes. In this report, we show that sE-selectin induced normal human peripheral blood monocyte migration in the nanomolar range in a concentration-dependent manner. Neutralization studies using RA human joint synovial fluids and anti-E-selectin antibody showed a mean 31% reduction in RA synovial fluid-mediated monocyte chemotaxis (p < 0.05), indicating that sE-selectin is a major monocyte recruiter in RA. Next, we investigated the role of tyrosine phosphorylation pathways in sE-selectin-induced monocyte chemotaxis. Human peripheral blood monocytes stimulated with sE-selectin showed a time-dependent increase in the tyrosine phosphorylation of a broad range of cellular proteins, predominantly in the molecular size range of Src family kinases (50–60 kDa) and mitogen-activated protein kinases (MAPKs). Western blot analysis of Src family kinases showed a time-dependent increase in Src, Hck, and Lyn phosphorylation. The pretreatment of monocytes with the Src inhibitor AG1879: 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo-[3,4-d]pyrimidine (PP2) prior to stimulation with sE-selectin markedly inhibited Hck and Lyn phosphorylation, whereas the phosphorylation of Src was partially inhibited. In addition, the sE-selectin stimulation of monocytes resulted in the increased phosphorylation of extracellular signal-related kinase (ERK1/2) and p38 MAPK. The pretreatment of monocytes with PP2 showed 89 and 83% inhibition of ERK1/2 and p38 MAPK phosphorylation, respectively. sE-selectin also showed a time-dependent activation of Ras kinase. Furthermore, the pretreatment of monocytes with PP2 completely inhibited sE-selectin-mediated monocyte chemotaxis. Taken together, our data demonstrate a novel function for sE-selectin as a monocyte chemotactic agent and suggest that sE-selectin might be mediating its biological functions through the Src-MAPK pathway.

The selectins are a family of three calcium-dependent lectins that mediate adhesive interactions between leukocytes and the endothelium during normal and abnormal inflammatory conditions such as rheumatoid arthritis (RA)1 or atherosclerosis (1, 2). E-selectin is a single-chain 115-kDa glycoprotein with a lectin-like N-terminal domain, an epidermal growth factor (EGF)-like motif, and a variable number of repeat units homologous to the consensus repeats of complement binding proteins (3). The lectin domain of E-selectin plays a major role in ligand recognition and binds to sialyl LeX on leukocytes as well as on endothelial cells (4–6). Soluble isoforms of these adhesion molecules are rapidly shed from the cellular surfaces on cellular activation (1). We have found elevated levels of soluble E-selectin (sE-selectin) in patients with RA (7). In contrast to the earlier hypothesis that soluble adhesion molecules serve an anti-inflammatory role, our laboratory has shown a unique role for sE-selectin as a proinflammatory, angiogenic mediator (8).

The infiltration of monocytes into the synovial tissue is a key factor in amplifying and perpetuating RA. The functions of elevated levels of sE-selectin and the mechanism of action of sE-selectin are still poorly understood. One of the primary mechanisms by which leukocytes are activated might be through the activation of protein tyrosine kinases at the cell surface (9, 10). The Src family of tyrosine kinases is activated during a number of biological functions including monocyte interaction with endothelial cells (11), endothelial cell differentiation (12), and cardiac motility (13). A number of pathways lead to the activation of mitogen-activated protein kinases (MAPKs). MAPKs are a family of 38–45 kDa proteins that exist in a dephosphorylated form in quiescent cells and can be activated in response to various growth factors and chemotactants (14, 15). MAPKs are activated by MAPK/ERK kinase (MEK), which in turn is activated by MEK kinase (MEKK). One of the extensively studied MEKKs is Raf kinase (16). Raf kinase is in turn activated by binding to Ras-GTP (17), and Src is an important upstream kinase linked to Ras-Raf activation (12, 18).

In this study, we examined the role and mechanism of action of sE-selectin in mediating monocyte recruitment. We report that sE-selectin is a potent chemotactic factor for monocytes, and its neutralization in RA synovial fluids, led to a significant decrease in RA synovial fluid-mediated monocyte migration. Furthermore, our results suggest that sE-selectin mediates signaling in monocytes through a Src-Ras-MAPK pathway. The

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1 The abbreviations used are: RA, rheumatoid arthritis; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; MN, mononuclear cells; PAGE, polyacrylamide gel electrophoresis; MLN, N-formyl-Met-Leu-Phe; sE-selectin, soluble E-selectin; EGF, epidermal growth factor; MEK, MAPK/ERK kinase; MEKK, MEK kinase; HBSS, Hank’s balanced salt solution; PP2, AG1879: 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine.
inhibition of Src kinase by the Src-specific inhibitor, PP2, abolished e-selectin-mediated monocyte chemotaxis. These results suggest that e-selectin mediates chemotaxis through the Src pathway, and this could thus be a potential target for modulating monocyte recruitment-driven diseases.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Recombinant human e-selectin was purchased from R&D Systems (Minneapolis, MN). e-selectin contained less than 0.1 ng of endotoxin/1 µg of protein content (as per the manufacturer). Accu-Prep was purchased from Accurate Chemical and Scientific Corporation (Westbury, NY). Percoll and Sigma’s balanced salt solution (HBSS) were obtained from Life Technologies, Inc. Orthovanadate, paranitrophenolphosphate, leupeptin, aprotinin, phenylmethylsulfonyl fluoride, dimethyl sulfoxide (Me2SO), bovine serum albumin, and N-formyl-Met-Leu-Phe (fMLP) were obtained from Sigma. Protease inhibitor mixture tablets were obtained from Roche Molecular Biochemicals. PP2 was purchased from Calbiochem. Mouse anti-human e-selectin monoclonal antibody RB11 (IgG1) was a gift from Biogen (Cambridge, MA). Mouse IgG, antibody (negative control) was purchased from Coulter (Hialeah, FL). Mouse monoclonal anti-phosphotyrosine antibody (4G10 clone) (Upstate Biotechnology, Inc.) was purchased from BD Biosciences (Franklin Lakes, NJ). Human monoclonal anti-human IgG (μE2/743) antibody was purchased from Accurate Chemical and Scientific Corporation (Westbury, NY). Polyclonal rabbit anti-human pSrc antibody (Tyr(P)418) and polyclonal rabbit anti-human p38 antibody was purchased from New England Biolabs (Beverly, MA). Polycarbonate filters with 5-µm pores were obtained from Poretics Corp. (Livermore, CA). Protein estimation reagents (BCA protein assay kit). Cell lysates were mixed at a 1:20 dilution with synovial fluid or buffer (negative control) and aliquots of cell lysates were heat shocked at 55 °C in 67 mM Tris at pH 6.7, 2% SDS, and 100 mM 2-mercaptoethanol for 10 min at room temperature. The protein content of different samples was quantitated using a bicinchoninic acid (BCA) protein assay kit. The viability of monocytes determined by trypan blue exclusion was found to be >98%, and the purity was >90%. The results are expressed as the mean and S.E. of three high power microscope fields were counted in each replicate well, and the results were expressed as cells per high power field. In synovial fluid neutralization studies, 1:2 diluted synovial fluid was preincubated with anti-e-selectin antibody (5 µg/ml) (Biogen) or corresponding control antibody (mouse IgG1) for 1 h at 37 °C. In the signaling inhibitor studies, monocytes (2.5 × 106 cells/ml) were preincubated with respective inhibitors (1 µM) for 1 h at 37 °C. After washing twice with HBSS containing calcium and magnesium, the cells were adjusted to 2.5 × 106 cells/ml and then assayed in response to different concentrations of e-selectin. The results are expressed as the mean and S.E. of three high power fields (×400) per replicate well, and each test group was assayed in quadruplicate. The incubation of monocytes with the Src inhibitor PP2 at 1 and 2 µM did not alter cell viability. For all assays, controls included HBSS (negative control), MLF (positive control), and Me2SO (a vehicle for the Src inhibitor).

**Cell Lysis and Immunoblotting**—Monocytes (1 × 107 cells/ml) were incubated in 24-well plates for 3 h in HBSS (with calcium and magnesium) prior to stimulation with e-selectin for various time points. At the end of each time period, supernatants were gently aspirated, and cells were lysed in extraction buffer containing 100 mM Tris, pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 20 mM Na3PO4, 2 mM Na2VO4, 1% Triton X-100, 10% glycerol, 0.1% SDS, 0.5% deoxycholate, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitors (protease inhibitor mixture tablets, 1 tablet/10 ml) (Roche Molecular Biochemicals). For experiments with signaling inhibitors, monocytes were preincubated with the respective inhibitor before activation with e-selectin. The protein content of different samples was quantitated using a bicinchoninic acid (BCA) protein assay kit. Cell lysates were mixed at a 1:1 with Laemmli’s sample buffer and boiled for 5 min. 15 µg of each sample was separated on 10% SDS-polyacrylamide gel electrophoresis. Separated proteins were transferred from the gel onto nitrocellulose membranes using a Tris-glycine buffer. To block nonspecific binding, membranes were incubated with 3% bovine serum albumin in Tris-buffered saline containing 0.1% Tween 20 (TBST) for 1 h at room temperature. The blots were incubated in respective primary antibody in TBST + 3% bovine serum albumin at 4 °C overnight. After washing with TBST, the blots were incubated with horseradish peroxidase-conjugated sheep anti-mouse IgG (1:10,000) or goat anti-rabbit IgG (1:10,000) for 45 min at room temperature. An enhanced chemiluminescence detection (ECL) system (Amersham Pharmacia Biotech) was used to detect specific protein bands. The different bands were then scanned and quantitated using an imaging densitometer (BioRad). The results are expressed as the mean and S.E. of three high power microscope fields were counted in each replicate well, and the results were expressed as cells per high power field. In synovial fluid neutralization studies, 1:2 diluted synovial fluid was preincubated with anti-e-selectin antibody (5 µg/ml) (Biogen) or corresponding control antibody (mouse IgG1) for 1 h at 37 °C. In the signaling inhibitor studies, monocytes (2.5 × 106 cells/ml) were preincubated with respective inhibitors (1 µM) for 1 h at 37 °C. After washing twice with HBSS containing calcium and magnesium, the cells were adjusted to 2.5 × 106 cells/ml and then assayed in response to different concentrations of e-selectin. The results are expressed as the mean and S.E. of three high power fields (×400) per replicate well, and each test group was assayed in quadruplicate. The incubation of monocytes with the Src inhibitor PP2 at 1 and 2 µM did not alter cell viability. For all assays, controls included HBSS (negative control), MLF (positive control), and Me2SO (a vehicle for the Src inhibitor).

**Statistical Analysis**—Data were analyzed using Student’s t test, and p values less than 0.05 were considered significant.

**RESULTS**

**sE-selectin Is Chemotactic for Monocytes in Vitro**—Our laboratory has shown that e-selectin levels are up-regulated in synovial fluids from patients with RA (7). To examine the hypothesis that e-selectin might be chemotactic for monocytes, we studied monocyte chemotaxis in response to e-selectin in a modified Boyden chamber. A representative assay from five experiments is shown in Fig. 1. e-selectin-induced chemotaxis in a concentration-dependent manner and was significantly higher at 10, 50, and 100 nM e-selectin concentrations as compared with the negative control (p < 0.05).

**Contribution of e-selectin to RA Synovial Fluid Chemotactic Activity for Monocytes**—We have shown earlier that RA synovial fluids from patients with RA (7) contain high levels of e-selectin. To examine the hypothesis that e-selectin might be chemotactic for monocytes, we studied monocyte chemotaxis in response to e-selectin in a modified Boyden chamber. A representative assay from five experiments is shown in Fig. 1. e-selectin-induced chemotaxis in a concentration-dependent manner and was significantly higher at 10, 50, and 100 nM e-selectin concentrations as compared with the negative control (p < 0.05).
Fig. 1. sE-selectin-mediated monocyte chemotaxis. Monocytes (2.5 × 10^5 cells/ml) in the upper chemotaxis chamber were incubated with various concentrations of sE-selectin in the lower chamber for 2 h at 37 °C. Results are expressed as the mean and S.E. of three high power fields (×400) per replicate well. Each test group was assayed in quadruplicate. This is a representative assay from five independent experiments. HBSS was used as a negative control, and fMLP (100 nM) was used as a positive control. sE-selectin showed a dose-dependent increase (p < 0.05) in monocyte chemotaxis as compared with the negative control HBSS. * represents a significant difference (p < 0.05) between the sE-selectin group and the negative control HBSS.

We next investigated the signaling pathways involved in the sE-selectin-mediated chemotaxis of normal PB monocytes. A 1:2 dilution of RA synovial fluid was preincubated with isotype-matched control antibody or anti-E-selectin antibody for 1 h at 37 °C. The incubation of RA synovial fluid with anti-E-selectin antibody significantly decreased monocyte chemotaxis (129 ± 3.2, 193 ± 4.4, and 90 ± 7.5) as compared with the isotype control antibody in the respective samples (225 ± 5.9, 270 ± 13.7, and 134 ± 4.1) (Table I). These data indicate that sE-selectin contributes significantly to the RA synovial fluid chemotactic activity for monocytes.

Signaling Mechanism Involved in sE-selectin-mediated Chemotaxis—We next investigated the signaling pathways involved in the sE-selectin-mediated monocyte chemotaxis. sE-selectin (50 nM) induced protein tyrosine phosphorylation in monocytes in a time-dependent manner showing two peaks. Western blot analysis showed a first peak at 30 s and a second peak at 30 min. Proteins phosphorylated at tyrosine residues corresponding to molecular sizes of 130–140, 70, 60–65, 50–55, 42–44, 35–40, and 25 kDa were detected (Fig. 2) by using a mouse monoclonal antibody (4G10), which specifically reacts with tyrosine phosphorylated proteins. Experiments shown in Fig. 2 were repeated five and three times, respectively, with essentially identical results.

As the predominant bands (50–65 kDa) observed with the 4G10 antibody corresponded to the molecular sizes of the Src family kinases, we next studied the role of Src family kinases Src, Hck, and Lyn in sE-selectin-mediated signaling in monocytes. We first performed a time course study of Src kinase activation followed by a Src inhibition study with the Src inhibitor PP2. Western blot analysis of activated Src with a phosphorylation state-specific antibody (Tyr(P)18) showed a biphasic activation of Src with the first peak at 30 s and a second peak at 30 min (Fig. 3). The pretreatment of monocytes with the Src inhibitor PP2 (1 μM) for 30 min prior to stimulation with sE-selectin partially inhibited Src phosphorylation (Fig. 4). We next studied the role of the two other Src family kinases (Hck and Lyn) in sE-selectin-mediated signaling in monocytes. Hck and Lyn kinases were immunoprecipitated from sE-selectin-stimulated monocytes by polyclonal rabbit anti-Hck or polyclonal rabbit anti-Lyn antibodies, respectively.

The activation of Hck and Lyn kinases was determined by subjecting these immunoprecipitates to SDS-PAGE and Western blotting with anti-phosphotyrosine antibody (4G10). Hck kinase showed a time-dependent increase in tyrosine phosphorylation (Fig. 5A), whereas Lyn kinase showed a minor peak at 30 s and a major peak at 30 min (Fig. 5B). The pretreatment of monocytes with PP2 markedly inhibited the phosphorylation of both Hck and Lyn kinases (Fig. 5). The total protein phosphorylation profile analysis in monocytes pretreated with PP2 prior to sE-selectin stimulation showed inhibition of the phosphorylation of proteins in the Src region (50–60 kDa) and MAPK region (35–45 kDa).

The activation of sE-selectin was determined by subjecting these immunoprecipitates to SDS-PAGE and Western blotting with anti-phosphotyrosine antibody (4G10). Hck kinase showed a time-dependent increase in tyrosine phosphorylation (Fig. 5A), whereas Lyn kinase showed a minor peak at 30 s and a major peak at 30 min (Fig. 5B). The pretreatment of monocytes with PP2 markedly inhibited the phosphorylation of both Hck and Lyn kinases (Fig. 5). The total protein phosphorylation profile analysis in monocytes pretreated with PP2 prior to sE-selectin stimulation showed inhibition of the phosphorylation of proteins in the MAPK region (35–45 kDa) in addition to the inhibition of Src family kinase region (60–70 kDa) (Fig. 2B).

### Table I

| Patients | Control antibody or neutralizing antibody | Mean cells (10⁴) high-power field | Suppression (%) | p* |
|----------|-----------------------------------------|----------------------------------|----------------|----|
| Patient 1 | SF + IgG<sub>1</sub> | 225 ± 5.9 | 34 | <0.05 |
| Patient 2 | SF + anti-E-selectin | 129 ± 3.2 | 18 | <0.05 |
| Patient 3 | SF + IgG<sub>1</sub> | 134 ± 4.1 | 40 | <0.05 |
| SF + anti-E-selectin | 90 ± 7.5 | 6 |

* RA SF samples were diluted 1:2 in Hanks’ balanced salt solution and assayed for normal PB monocyte chemotaxis. Results represent mean ± S.E. of 12 high power fields (×400) per sample.

* Percent suppression was determined as compared with matched isotype control antibody.

* By Student’s t test.
To test the hypothesis that the sE-selectin signal from Src family kinases might be acting through MAP kinases, we studied the time course of extracellular signal-related kinase (ERK1/2) and p38 MAPK activation in monocytes. ERK1/2 showed a biphasic activation profile as observed with Src kinase with the first peak occurring at 1 min and the second peak at 30 min (Fig. 6). However, p38 MAPK did not show phosphorylation until 5 min after stimulation and showed a single peak at 30 min (Fig. 7). Next, we studied the role of the Src inhibitor PP2 on ERK1/2 and p38 MAPK activation. The pretreatment of monocytes with the Src-specific inhibitor PP2 markedly decreased Src phosphorylation by sE-selectin. This blot is representative of three independent experiments.

FIG. 3. Time-dependent activation of Src in monocytes by sE-selectin. Monocytes were stimulated with sE-selectin (50 nM) for various time points as indicated in the figure (m, minutes). Cell extracts were prepared with cell lysis buffer, and the protein content of each sample was quantitated. 5 μg of each sample was subjected to 10% SDS-PAGE and probed with rabbit polyclonal anti-pSrc antibody (0.5 μg/ml). sE-selectin induced the tyrosine phosphorylation of Src in a time-dependent manner showing a biphasic activation pattern. The first activation peak occurred at 30 s, and the second peak occurred at 30 min. This blot is representative of three independent experiments.

FIG. 4. Inhibition of sE-selectin-mediated Src activation by the Src-specific inhibitor PP2. Monocytes were pretreated with the Src-specific inhibitor PP2 (1 μM) for 30 min at 37 °C before being stimulated with sE-selectin for different time points. Cell extracts were prepared with lysis buffer at each time point, and the protein concentration in each sample was quantitated. 15 μg of each sample was subjected to 10% SDS-PAGE and probed with rabbit polyclonal anti-pSrc antibody (0.5 μg/ml). The pretreatment of monocytes with the Src-specific inhibitor PP2 markedly decreased Src phosphorylation by sE-selectin. This blot is representative of three independent experiments.

FIG. 5. Hck and Lyn activation in monocytes stimulated with sE-selectin. Monocytes, untreated or preincubated with 1 μM PP2 for 30 min, were stimulated with sE-selectin (50 nM). Cell extracts were prepared at various time points, and the protein concentration in each sample was quantitated. 15 μg of each sample was subjected to 10% SDS-PAGE and probed with rabbit polyclonal anti-pHck or anti-pLyn antibody. sE-selectin induced the tyrosine phosphorylation of Hck and Lyn in a time-dependent manner, which was markedly inhibited by pretreatment with PP2. These results are representative of three independent experiments.

FIG. 6. ERK1/2 activation in monocytes stimulated with sE-selectin. Monocytes were stimulated with sE-selectin (50 nM) at various time points as indicated (m, minutes). Cells were extracted with cell lysis buffer, and the protein content of each sample was quantitated. 15 μg of each sample was subjected to 10% SDS-PAGE and probed with rabbit polyclonal anti-pERK1/2 antibody. sE-selectin stimulation of monocytes induced enhanced the tyrosine phosphorylation of ERK1/2 in a time-dependent manner as compared with the control nonstimulated monocytes. This blot is representative of three independent experiments.

sE-selectin is a glycoprotein and contains an EGF-like motif (3), so we tested whether sE-selectin mediates its action through the EGF receptor. Our results show that sE-selectin does not induce the phosphorylation of the EGF receptor (data not shown). We also studied the phosphorylation profile of focal adhesion kinase, a substrate for the Src family kinases. However, we failed to detect the phosphorylation of focal adhesion kinase in sE-selectin-stimulated monocytes (data not shown). These data indicate that in contrast to Src family kinases (Src, Hck, and Lyn) and MAPKs (ERK1/2 and p38), the EGF receptor and focal adhesion kinase pathways are not involved in sE-selectin-mediated monocyte signaling.

A number of studies have shown that Raf kinase is a predominant MEK kinase (16, 23) that in turn is activated by binding to Ras-GTP (17). Src family kinases have also been shown to be linked to MAPK through Ras/Raf kinases (12, 18).
To study the activation of Ras/Raf kinases, we first immunoprecipitated activated Ras with a Raf-1-Ras binding domain conjugated to agarose beads and subsequently probed with mouse monoclonal anti-Ras antibody. sE-selectin stimulation of monocytes induced the enhanced tyrosine phosphorylation of p38 MAPK in a time-dependent fashion as compared with the control nonstimulated monocytes. This blot is representative of three independent experiments.

Next, to examine the functional role of Src kinase in sE-selectin-induced monocyte chemotaxis, we performed monocyte chemotaxis in the presence and absence of the Src inhibitor PP2 (1 μM) at 37°C prior to performing the chemotaxis assay. fMLP served as the positive control, and HBSS served as the negative control. Fig. 11 shows a representative assay from three independent experiments. The pretreatment of monocytes with the Src-specific inhibitor PP2 markedly inhibited monocyte chemotaxis as compared with sE-selectin alone, thereby suggesting that sE-selectin could be mediating monocyte chemotaxis through the Src pathway.

DISCUSSION
Monocyte recruitment into the synovial tissue is a key step in RA pathogenesis because activated monocytes and/or macrophages can function as antigen-presenting cells, and these cells also have the ability to secrete a variety of inflammatory mediators including prostaglandins (24), nitric oxide (25), interleukin-1, tumor necrosis factor-α (26), interleukin-8 (27), monocyte chemotactic protein-1 (28), and macrophage inflammatory protein-1α (29). We and others have reported a number of putative monocyte/macrophage chemoattractants in the RA synovial fluid, such as monocyte chemotactic protein-1 and macrophage inflammatory protein-1α (29, 30), which may be involved in monocyte/macrophage recruitment into the RA synovial tissue.

In this report, we show that sE-selectin is a potent chemoattractive agent for monocytes in the nanomolar range. We have
that the cross-linking of endothelial cell surface E-selectin with inflammatory leukocyte recruitment (31). Hu for monocytes in the RA synovial fluid. Thus, these results represent a significant difference (p < 0.05) between the sE-selectin group and negative control. # represents a significant inhibition (p < 0.05) of chemotaxis in the sE-selectin + PP2 (Src inhibition) group as compared with sE-selectin alone.

previously shown elevated levels of sE-selectin (in the nanomolar range) in the RA synovial fluid (7). Thus, it is likely that at the concentrations found in the RA joints, sE-selectin might be acting in large part to recruit and activate monocytes. To address the contribution provided by the sE-selectin to RA synovial fluid chemotactic activity for monocytes, we immunodepleted RA synovial fluid sE-selectin by using neutralizing mouse monoclonal anti-E-selectin antibodies. The neutralization of RA synovial fluid sE-selectin significantly decreased monocyte chemotaxis (mean of 31%, p < 0.05) as compared with the matched isotype control antibody. Thus, these results suggest a novel function for sE-selectin as a chemotactic factor for monocytes in the RA synovial fluid.

Recent studies have shown that intact, transmembrane E-selectin can transduce signals in endothelial cells during inflammatory leukocyte recruitment (31). Hu et al. (23) showed that the cross-linking of endothelial cell surface E-selectin with antibodies leads to the up-regulation of mRNA for c-fos, an early response gene, through the Ras-Raf-MAPK pathway. However, we are not aware of any studies regarding the signaling pathway used by sE-selectin to mediate its biological function. In this regard, we investigated sE-selectin-mediated signaling in normal PB monocytes. sE-selectin induced the tyrosine phosphorylation of a number of monocyte proteins corresponding to molecular sizes of 130–140, 70–74, 60–65, 50–55, 42–44, 35–40, and 20–25 kDa in a time-dependent manner. Marked increases in tyrosine phosphorylation were detected as early as 30 s after stimulation and peaked at 30 min, which is consistent with a typical time course of protein tyrosine phosphorylation (13, 32). We next investigated the role that Src family kinases play in sE-selectin-mediated signaling. The Src family of tyrosine kinases is reported to be activated during a number of biological phenomena including monocyte adherence to endothelial cells (11), integrin-mediated signaling in monocytes and/or macrophages (33), endothelial cell differentiation (12), and cardiac contractility (13). In our studies, sE-selectin up-regulated the phosphorylation of Src family kinases (Src, Hck, and Lyn) in a time-dependent manner. The pretreatment of monocytes with PP2 for 30 min prior to sE-selectin stimulation partially inhibited Src phosphorylation, whereas Hck and Lyn phosphorylation was markedly inhibited. These results indicate that the Src family kinases may be an important mediator in sE-selectin-mediated signaling in monocytes.

We next studied the role of PP2 on the total cellular phosphotyrosine pattern. The pretreatment of monocytes with PP2 prior to stimulation with sE-selectin showed the partial inhibition of a 60-kDa band. However, other Src family kinases (50–60 kDa) were substantially inhibited, thus corroborating our other findings that PP2 has a partial effect on sE-selectin-mediated Src phosphorylation, whereas it markedly inhibited Hck and Lyn kinases. In addition to the Src family kinases, PP2 also showed substantial inhibition of the tyrosine phosphorylation of proteins in the MAPK region (35–45 kDa). MAPK is a key signaling central point at which a number of pathways converge (34). MAPK is involved in a number of basic physiological phenomena, including cell migration, cell cycle, and apoptosis, in addition to playing an important role in the signaling of a number of important genes such as chemokines (35) and adhesion molecules (36, 37). We next determined whether sE-selectin-mediated signaling involves the activation of MAPK. sE-selectin-stimulated monocytes showed the time-dependent phosphorylation of ERK1/2 and p38 MAPK. ERK1/2 activation was observed as early as 30 s and persisted until 30 min. However, p38 activation was detectable from 5 min onwards. To test the hypothesis that the signal from Src might be going through MAPK, we pretreated monocytes with the Src inhibitor PP2 and then studied the phosphorylation of ERK1/2 and p38. Pretreatment with the Src inhibitor (PP2) showed 89 and 83% inhibition of ERK1/2 and p38, respectively, at 30 min after sE-selectin stimulation. These results suggest that MAPKs (ERK1/2 and p38) are activated in monocytes by sE-selectin through the Src kinase pathway. Schmid-Aliena et al. (38) have demonstrated that microtubule depolymerization by colchicine in human monocytes induces the selective production of interleukin-1 through the Src, Ras, Raf-1, and MAPK pathway. A similar signaling cascade involving Src, Ras, and MAPK was reported by Jalali et al. (35) in vascular endothelial cells stimulated with shear stress.

Raf kinase is one of the best characterized MEKks (16). Raf kinase is activated by binding to Ras-GTP, which then phosphorylates MEK. Ras kinase has been shown to be activated by Src kinase (12, 35). To test whether Ras and Raf kinases are also activated during the sE-selectin activation of monocytes, we immunoprecipitated Ras with the Raf-1-Ras binding domain linked to agarose beads and then probed with mouse monoclonal anti-Ras antibody. sE-selectin stimulation showed a time-dependent activation of Ras, thereby suggesting a possible link between Src and MAPK in sE-selectin signaling through Ras-Raf kinase.

We next determined whether this Src-MAPK signaling cascade has functional relevance and whether it is essential for sE-selectin-mediated monocyte chemotaxis. PB monocytes were pretreated with the Src-specific inhibitor PP2 at a concentration of 1 μM for 30 min at 37°C before performing chemotaxis in response to sE-selectin. PP2 completely blocked sE-selectin-mediated chemotaxis. These data suggest that sE-selectin uses the Src pathway to mediate monocyte chemotaxis.

In summary, we have demonstrated a novel function for sE-selectin as a monocyte chemotactic agent. sE-selectin accounted for a significant proportion of the monocyte chemotactic activity in the RA synovial fluid. We have further shown that sE-selectin uses the Src-Ras-MAPK signaling pathway to mediate its biological function. Thus, sE-selectin may be a potent recruiter of monocytes in the RA joint and may contrib-
ute to RA pathogenesis through the Src-Ras-MAPK signaling pathway.

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