Retrograde Inflammatory Signaling from Neutrophils to Endothelial Cells by Soluble Interleukin-6 Receptor Alpha

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Abstract

Endothelial cells initiate the inflammatory response by recruiting and activating leukocytes. IL-6 is not an agonist for this, but we found soluble IL-6 receptor α-subunit (IL-6Rα), with their constitutive IL-6 synthesis, stimulated endothelial cells to synthesize E-selectin, intracellular adhesion molecule-1, vascular cellular adhesion molecule-1, IL-6, and IL-8, and to bind neutrophils. Neutrophils express significant amounts of IL-6Rα and upon stimulation shed it: this material activates endothelial cells through a newly constituted IL-6 receptor. Retrograde signaling from PMN activated in the extravascular compartment to surrounding endothelial cells will recruit more and a wider variety of leukocytes. The limiting signal is a soluble receptor, not a cytokine. (*J. Clin. Invest.* 1997. 100:2752–2756.) Key words: inflammation • leukocyte • E-selectin • interleukin-8 • chemokine

Introduction

Endothelium is the gatekeeper of the inflammatory response: it attracts and activates leukocytes when endothelial cells are appropriately stimulated (1). Marginated leukocytes rapidly migrate to the extravascular space where exposure to additional stimuli causes release of reactive oxygen species and proteolytic enzymes (2, 3). One consequence of the destructive environment surrounding activated PMN is the shedding of surface proteins such as L-selectin (4) and TNF receptors (5). The functional consequence of this self-induced loss of surface proteins is not clear as this occurs after migration into the extravascular compartment and full activation.

Neutrophils and monocytes express receptors for IL-6 consisting of a cytokine-binding alpha subunit (IL-6Rα)[1] and a homodimer of transmembrane, signaling gp130 subunits (6). Receptors for other members of the IL-6 family also contain the signaling gp130 subunit and combinations of cytokine-specific α-subunits and gp130-related subunits. Monocytes and COS-7

cells expressing recombinant IL-6Rα shed a portion of this receptor in response to the pleiotrophic agonist PMA in a form able to bind cytokine and stimulate gp130 signaling (7, 8). IL-6 alone does not activate endothelial cells (although there is a single report to the contrary [9]) even though oncostatin M, a related family member whose receptor contains gp130 (10), does activate the inflammatory response of these cells (11). This result would be expected should endothelial cells lack IL-6Rα. Since truncated, soluble IL-6Rα can confer IL-6 sensitivity to cells containing gp130 (12), including endothelial cells (13), we determined whether a complex of IL-6 and sIL-6Rα activated the inflammatory response of human endothelial cells. We found this complex did stimulate the inflammatory response. However, more striking was that the addition of sIL-6Rα alone activated endothelial cells: it appears constitutive endothelial cell IL-6 expression was sufficient to form an active complex with exogenous soluble receptor. Endothelial cell activation depended on the concentration of sIL-6Rα, showing the solublized receptor was the limiting component of the signaling process. This observation is relevant as we found PMN exposed to the bacterial peptide FMLP shed IL-6Rα in sufficient quantities to activate the inflammatory response of endothelial cells. These results suggest that ablumenal neutrophil degranulation and cell surface protein shedding could send a retrograde signal back through the endothelium to recruit more inflammatory cells from the circulation.

Methods

Materials. HBSS and M199 were from Whittaker Bioproducts (Walkersville, MD) and human serum albumin was from Miles Laboratories (Elkhart, IN.) Tissue culture plates were from Costar Data Packaging Corp. (Cambridge, MA). Polyclonal rabbit anti–human IL-8, IL-6Rα, gp130, and E-selectin (BBA-8) antibodies , and recombinant human oncostatin M, gp130, sIL-6Rα, and TNF were from R&D systems (Minneapolis, MN). Endogen (Boston, MA) supplied polyclonal rabbit anti–human IL-6 and Biosource International (Camarillo, CA) supplied recombinant human IL-6. Anti–human vascular cellular adhesion molecule-1 (VCAM-1), intracellular adhesion molecule-1 (ICAM-1), and IL-6 (see Fig. 2) were from PharMingen (San Diego, CA.) Bachem (Torrence, CA) supplied IL-6$_{88-121}$. ECL Western blotting reagents were purchased from Amersham Corp. (Arlington Heights, IL). All secondary antibodies were obtained from Biosource International. Other reagents were from Sigma Chemical Co. (St. Louis, MO).

Cell isolation and culture. Human umbilical vein endothelial cells were cultured in 24- or 35-mm multiwell plates as described (14). Only monolayers of primary cultures that were tightly confluent (24 h postconfluence) were used for these studies. Routine cultures show < 1% contamination with CD45$^+$ cells. Neutrophils were isolated from EDTA-anti–coagulated human blood and labeled with ^{111}In-oxine for adhesion to a gelatinized surface or activated endothelial cells as described (15).

Endothelial cell activation. Medium was removed from postconfluent endothelial cells, and agonist in Hank's balanced salt solution

1. *Abbreviations used in this paper:* IL-6Rα, IL-6 receptor α-subunit; sIL-6Rα, soluble IL-6Rα.

containing 0.5% human serum albumin (HBSS/A) was added for the stated time. Polymyxin B did not affect any parameter we examined, so it was routinely used at 10 μg/ml during cell stimulation. When the effect of anti–IL-6, anti-gp130, or IL-6$_{88–121}$ was examined, they were preincubated with endothelial cells for 90 min. In some experiments medium was removed from the monolayers and they were incubated for 24 h in HBSS/A before the cells were washed three times with HBSS/A. Supernatants were removed from incubated monolayers after 4 h for ELISA determination of IL-8 or IL-6, while the monolayers were used to quantitate inflammatory protein synthesis or PMN adhesion.

ELISA, flow cytometry, and Western blotting. Material for Western blots was collected by solubilizing monolayers with boiling Laemmli (16) sample buffer without β-mercaptoethanol. Proteins were electrophoretically separated under nonreducing conditions in 7.5% SDS polyacrylamide gels and then transferred to Immobilon membranes (Millipore, Bedford, MA). The membrane was blocked with 5% nonfat milk in Tris-buffered saline with 0.05% Tween-20 (TBST), and probed with 1 μg/ml anti–E-selectin monoclonal antibody. Horseradish peroxidase-conjugated streptavidin (1:5,000) in the presence of 100 μg/ml nonimmune rabbit IgG in TBST was detected by ECL. IL-6 and IL-8 were quantitated from endothelial cell supernatants by sandwich ELISA with polyclonal rabbit anti–human antibody; detection used biotinylated rabbit anti–human IL-6 or IL-8, and streptavidin-conjugated horseradish peroxidase (17). Endothelial cells were released from the culture dish with a brief trypsin treatment, washed in goat serum, and stained for surface ICAM-1 or VCAM-1 (17).

PMN shedding. PMN (5.5×10^6/ml) were incubated (30 min; 37°C) with 10^{-6} M FMLP, PMA (10^{-6} M), or buffer and then centrifuged (1,000 *g*, 5 min). Anti–IL-6Rα (25 μg/ml) or soluble gp130 (10 μg/ml) was added to some supernatant aliquots (30 min preincubation at 37°C) to block receptor function. In addition, some monolayers were pretreated for 30 min at 37°C with anti-gp130 (50 μg/ml). Endothelial cells were incubated for 4 h at 37°C with PMN supernatants (1 ml/well) before E-selectin expression was determined by Western blotting.

Results

sIL-6Rα activates endothelial cells to bind PMN. Oncostatin M, but not its family member IL-6, activated endothelial cells to bind quiescent PMN (Fig. 1 *A*). This shows that endothelial cells possess functional gp130, which is common to both oncostatin M and IL-6 receptors (10), and suggests these cells lack the IL-6Rα subunit of the IL-6 receptor that binds IL-6. We analyzed surface IL-6Rα expression by flow cytometry and mRNA levels by reverse transcriptase-PCR, which confirmed that the insensitivity to IL-6 reflects a lack of this receptor component (see Fig. 4 *A*). Consistent with this, we found that addition of a complex of IL-6 and a truncated, soluble form of the alpha subunit (sIL-6Rα) activated endothelial cell-dependent PMN adhesion (Fig. 1 *A*). Polymyxin B did not affect any of these responses, so endotoxin was not responsible for this stimulation. In a surprising result, we found that sIL-6Rα alone was just as effective an agonist as the preformed complex of cytokine and its soluble receptor (Fig. 1 *A*). Moreover, we found that PMN adhesion to the activated endothelial cells was dependent on the concentration of sIL-6Rα (Fig. 1 *B*). Thus the soluble receptor was the limiting component under these conditions. PMN adhesion was an endothelial cell-dependent process as sIL-6Rα or IL-6 failed to stimulate PMN in the absence of endothelial cells (control, 6±2%; 1 μg/ml sIL-6sR, 6±1%; 100 ng/ml IL-6, 10±3%; 10^{-8} M FMLP, 38±0% PMN bound to a gelatinized surface as a measure of CD11b/CD18 effectiveness [18]).
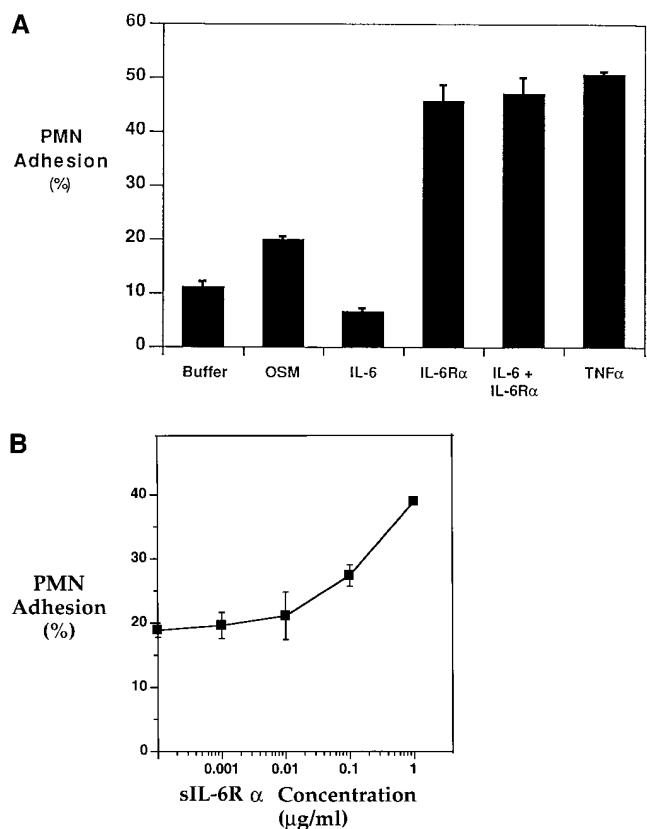
Figure 1. sIL-6Rα activates endothelial cell-dependent PMN adhesion. (*A*) Endothelial cells were incubated with oncostatin M (100 ng/ml), IL-6 (100 ng/ml), sIL-6Rα (1 μg/ml), both IL-6 and sIL-6Rα, or TNFα (500 U/ml) for 4 h. ^{111}In-PMN adhesion to the monolayers during a 5-min period was quantitated as a percentage of total unactivated PMN added. (*B*) Endothelial cells were incubated with the stated concentrations of sIL-6Rα for 4 h and ^{111}In-PMN adhesion was determined as before. The values shown are mean and range of duplicate determinations and are representative of two experiments.

sIL-6Rα activates endothelial cell inflammatory protein synthesis. We found (Fig. 2 *A*) that sIL-6Rα induced the expression of the PMN-binding protein E-selectin. Induction of E-selectin synthesis and accumulation was blocked by an inhibitory IL-6 antibody, by an antibody to the gp130 receptor subunit common to IL-6 and oncostatin M receptors, and by a synthetic peptide fragment of IL-6 that competitively blocks IL-6 binding to the soluble receptor (19). The anti-gp130 antibody also suppressed oncostatin M, but not TNFα, stimulation of E-selectin synthesis (not shown). We found that sIL-6Rα also stimulated the expression of the proinflammatory counterligand ICAM-1 (Fig. 2 *B*), and that it stimulated surface expression of the monocyte-binding protein VCAM-1 (Fig. 2 *C*). sIL-6Rα also induced the synthesis of the inflammatory C–X–C chemokine IL-8 (Fig. 3 *A*), and, interestingly, it induced the synthesis of more IL-6 (Fig. 3 *B*). We conclude sIL-6Rα activates endothelial cell inflammatory functions through endothelial cell-derived IL-6 and the gp130 subunits of the IL-6 holoreceptor normally present as a component of the oncostatin M receptor.

PMN shed IL-6Rα upon activation. Resting human neutrophils, unlike endothelial cells (Fig. 4 *A*), abundantly express IL-6Rα on their surface (Fig. 4 *B*), but its expression is labile.
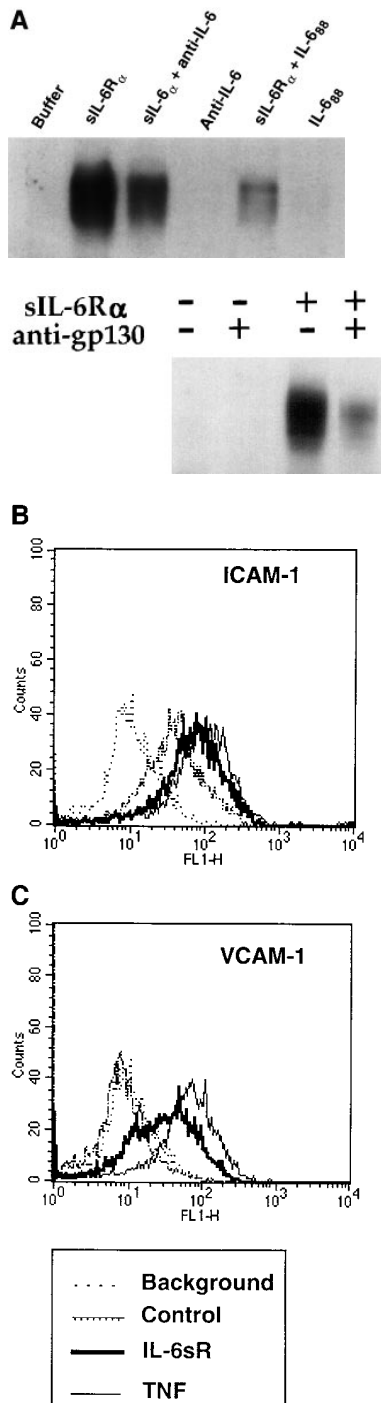
Figure 2. sIL-6Rα stimulates endothelial cell expression of adhesion proteins. (*A*) E-selectin expression. Endothelial cells were preincubated with buffer, anti-gp130 (50 μg/ml), anti–IL-6 (PharMingen, 1 μg/ml), or the synthetic IL-6$_{88-121}$ fragment (5 μg/ml) for 90 min and then incubated in the presence of the reagents with or without sIL-6Rα (1 μg/ml) for 4 h. E-selectin was assayed by immunoblotting as described in Methods. (*B*) Expression of ICAM-1 and (*C*) VCAM-1 by control or sIL-6Rα–activated endothelial cells was analyzed by flow cytometry using nonimmune mouse IgG to determine nonspecific staining. The data presented are one of two experiments with similar results.

not account for this as FMLP itself did not induce E-selectin synthesis. Anti–IL-6Rα antibody, anti-gp130 antibody, or competing soluble gp130 inhibited over half the E-selectin expression (Fig. 5). Ligation of the receptor complex by anti-gp130 evoked a small increase in E-selectin expression, while soluble gp130, which is shed in a less facile fashion than the alpha subunit (21), failed to induce E-selectin synthesis. Thus a functional IL-6 receptor can be assembled on endothelial cells using solubilized alpha subunits shed from activated neutrophils, and this de novo receptor stimulates the inflammatory response of endothelial cells.

Discussion

IL-6 is considered to be an inflammatory cytokine, although it was not known to participate in the earliest phases of the inflammatory reaction where endothelial cells, PMN, and monocytes interact. Endothelial cells do not respond to IL-6 as they lack the cytokine-binding alpha subunit of the receptor ([22], Fig. 4 *A*). A remarkable property of the IL-6 receptor is that the nonsignaling alpha subunit can be exogenously supplied in

Figure 3. sIL-6Rα induces cytokine secretion from endothelial cells. Endothelial cells were incubated with sIL-6Rα (1 μg/ml), TNF (100 U/ml), or oncostatin M (1 μg/ml) for 4 h. Supernatants were collected and (*A*) IL-8 and (*B*) IL-6 concentrations were assayed by sandwich ELISA. Values are mean and range of duplicate determinations, and are representative of three experiments.
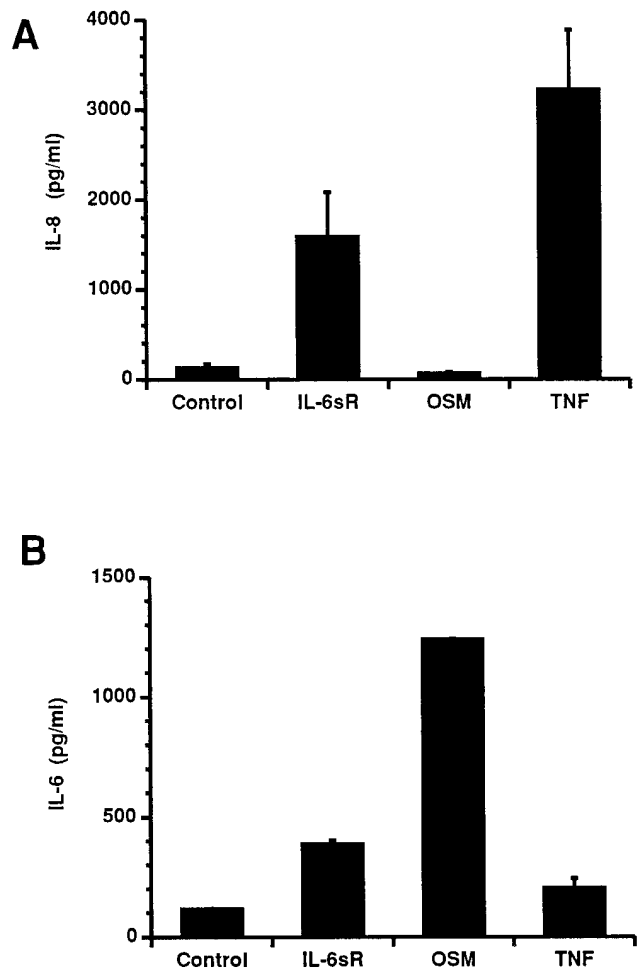
We found that expression of this receptor subunit was significantly reduced after exposure to FMLP or phorbol myristate acetate. This loss of surface IL-6Rα correlated with a decrease in surface L-selectin expression (20), suggesting sIL-6Rα may be shed from activated PMN in a similar fashion (8).

Shed sIL-6Rα activates endothelial cells. We determined whether sIL-6Rα was shed from activated neutrophils in sufficient quantities to stimulate endothelial cells. We treated PMN with buffer or FMLP for 30 min, removed the PMN, and then exposed endothelial cell monolayers to these supernatants. Supernatants from FMLP-activated neutrophils stimulated endothelial cell E-selectin synthesis, while supernatants from unstimulated neutrophils did not (Fig. 5). FMLP carryover did
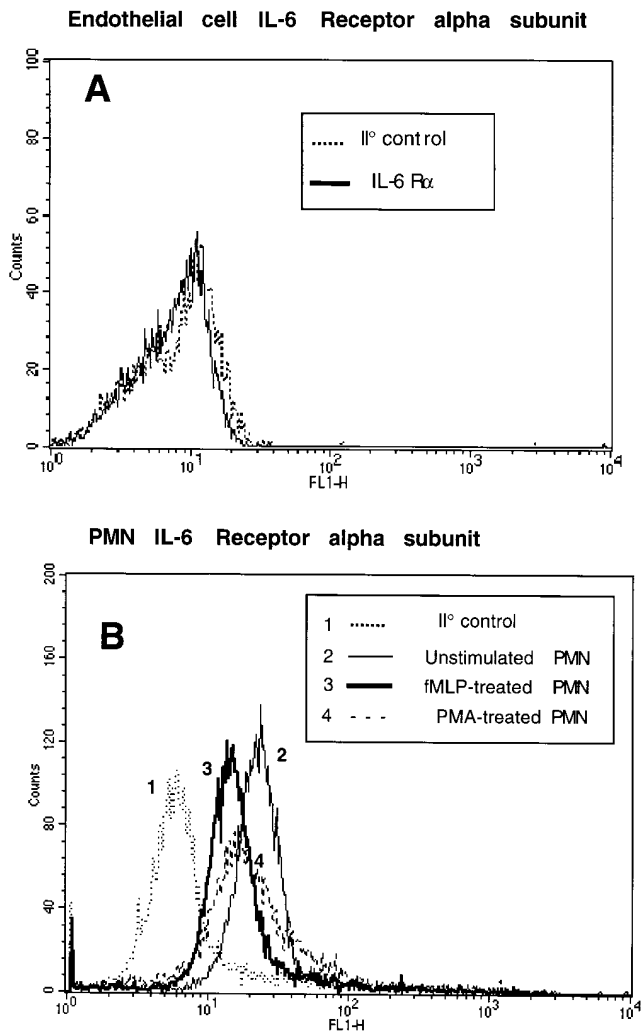
Endothelial cell IL-6 Receptor alpha subunit

A

...... II° control

—— IL-6 Rα

PMN IL-6 Receptor alpha subunit

B

| 1 | ...... | II° control |
2	——	Unstimulated PMN
3	——	fMLP-treated PMN
4	- - -	PMA-treated PMN

Figure 4. IL-6Rα is shed from activated PMN. (*A*) Endothelial cells. Endothelial cells were removed from their growth plates and stained with nonimmune control or anti–IL-6Rα for flow analysis. (*B*) PMN were treated with buffer, PMA (10^{-6} M), or FMLP (1 μM) for 30 min and surface IL-6Rα was analyzed by flow cytometry using goat anti–human anti–IL-6Rα. Background antibody staining was determined with nonimmune goat IgG.

a soluble form and still confer specific cytokine sensitivity (12, 23). We found that soluble IL-6Rα recruited gp130 from the very large (> 110,000 receptors per cell [24]) pool of endothelial cell oncostatin M receptors and allowed it to mimic proinflammatory effects of TNFα. An unexpected finding in this regard was that sIL-6Rα did not require the addition of exogenous IL-6 to activate these cells. Endothelial cell-derived IL-6, which is constitutively synthesized at low levels, was essential for this activation, but the most significant aspect of this is that the inflammatory response varied as a function of sIL-6α concentration. Thus the agonist to which primary, but not multiply passed (22), endothelial cells respond in a graded fashion is the soluble receptor, not the cytokine. Previously, the role of sIL-6Rα has appeared to be mundane as its transgenic overexpression simply increases the half-life of IL-6 in the circulation and sensitizes animals to an acute phase response (25). Our data show that it can function as a true proinflammatory cytokine where its levels control endothelial cell function.

Inflammatory signaling is a vectoral process where stimulated endothelial cells recruit leukocytes from the circulation to the extravascular compartment. To this end endothelial cells synthesize and secrete several members of the C–X–C and C–C class of chemokines that activate neutrophils and monocytes, respectively. Few PMN products can reverse the flow of information and activate endothelial cells: although eventually cytokines synthesized by PMN (26) or massive free radical production by activated PMN (27, 28) might do so. Here we show that IL-6Rα is rapidly shed from FMLP-activated PMN in sufficient quantities to allow activated PMN to efficiently stimulate endothelial cells. Proteolytic shedding of external surface proteins after activation serves uncertain purposes (29), although modification of adhesion is clearly one such function (4). The IL-6 receptor is shed from monocytes stimulated with the potent tumor promoter PMA and, as we show here, is shed from PMN after activation by a relevant, pathophysiologic agonist, FMLP. The role of IL-6 receptors on neutrophils has not been well defined, although it weakly induces platelet-activating factor synthesis (30) and weakly synergizes with TNF to enhance the respiratory burst (31). One outcome of our data is that the target of IL-6 signaling is not just the PMN expressing the IL-6 receptor, but also adjacent cells. This receptor then may be a sensitive environmental reporter that reverses signal flow by enabling proximal endothelial cells to respond to activated PMN.

Soluble IL-6Rα may be a particularly relevant proinflammatory mediator following bacterial invasion. PMN exposed to the bacterial product FMLP (above), or monocytes exposed to pore-forming exotoxins (32) or bacterially derived metalloproteinases (33) shed their IL-6 receptor. Also, circulating sIL-6Rα increases during sepsis (34), HIV infection (35), and reperfusion injury (36), although a part of this is due to mRNA alternative splicing (37). Recent data show the influx of PMN into inflamed joints correlates with the increased concentration of sIL-6Rα in synovial fluid (38), and that sIL-6Rα can act as a paracrine mediator localized to its site of generation (39). We show sufficient amounts of sIL-6Rα, and potentially other unidentified endothelial cell agonists, can be released from activated PMN to induce the endothelial cell inflammatory re-

PMN Supernatant	C	f	-	f	f	f	f	-	-	-
fMLP			+							
α-gp130				-	+			+		
α-IL-6Rα				-		+			+	
sol-gp130				-			+			+
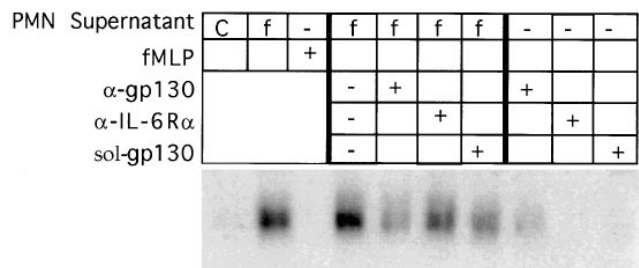
Figure 5. sIL-6Rα shed from activated PMN stimulates endothelial cell E-selectin synthesis. Supernatants from buffer-treated PMN (*C*) or FMLP-treated PMN (*f*) were prepared as described in Methods. An aliquot of supernatants from stimulated or unstimulated PMN was incubated (50 μg/ml) with anti–IL-6Rα or soluble gp130 (*sol-gp130*) for 60 min. Meanwhile endothelial cells were preincubated with anti-gp130 (50 μg/ml) or buffer for 90 min. The monolayers were then incubated (in the continued presence of the antibody if used) with buffer, PMN supernatants, or FMLP (1 μM) for 4 h. Cell lysates were prepared and E-selectin expression was determined by immunoblotting. The data are representative of two experiments.

sponse. This will occur after extravasated PMN find appropriate stimuli in the extravascular compartments, so pathfinding PMN can locally recruit more leukocytes from the circulation through retrograde activation of adjacent endothelial cells. Consistent with this model, the absence of IL-6 in homozygous knockout mice reduces the number of leukocytes recruited by extravascular stimuli by half (22). This shows that IL-6 and its receptor are not unique proinflammatory mediators, but do have major effects on the early steps in inflammation by assisting and strengthening the primary response to inflammatory stimuli.

Acknowledgments

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