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Tumour necrosis factor-alpha but not interleukin-1 induces polymorphonuclear leucocyte migration through fibroblast layers by a fibroblast-dependent mechanism

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SUMMARY

Interleukin-1 (IL-1) and tumour necrosis factor-alpha (TNF-α) both induce polymorphonuclear leucocyte (PMNL) infiltration into tissues and they have a synergistic action in this respect. We and others have observed that IL-1α and TNF-α induce ^{51}Cr-labelled PMNL migration across monolayers of umbilical vein endothelium via an endothelial cell-dependent mechanism. Here we investigated the interaction of PMNL with fibroblasts, since PMNL probably encounter such cells in many tissues once they traverse the vascular wall. TNF-α, but not IL-1α, was found to activate fibroblast monolayers, grown on polycarbonate filters, to stimulate PMNL transfibroblast migration. This was a time- and fibroblast-dependent process which required fibroblast protein synthesis, as indicated by inhibition with cycloheximide. The effect of TNF-α was not related to fibroblast chemotactic factor production (primarily IL-8), or to ICAM-1 up-regulation, since IL-1 was as active as TNF-α in this respect, without activating fibroblasts to support PMNL transfibroblast migration. Antiserum to IL-8, present during the assay, did not inhibit PMNL migration across the monolayers. The PMNL migration was highly dependent on the function of both CD11a (LFA-1) and CD11b (MAC-1) PMNL adhesion molecules, since monoclonal antibodies to either inhibited migration by about 80%. The results suggest a distinct activation by TNF-α of fibroblasts to facilitate PMNL migration through fibroblast barriers. These findings may in part account for the synergistic action of IL-1 and TNF-α in inducing extravascular accumulation of PMNL during inflammation.

INTRODUCTION

Acute inflammatory reactions are usually characterized by polymorphonuclear leucocyte (PMNL) margination in post-capillary venules, emigration across the vascular endothelium, and accumulation in the extravascular connective tissue. This margination and emigration is in part induced by chemotactic factors, which exert effects primarily on PMNL, stimulating their adhesion and migration. Recently, several cytokines which also induce PMNL infiltration into tissues *in vivo*, most notably interleukin-1 (IL-1) and tumour necrosis factor-alpha (TNF-α),[1,3] have been shown to exert their effects via endothelial-dependent mechanisms (reviewed in ref. 4). These molecules activate endothelium, increasing its adhesiveness for PMNL *in vitro*, and we and others have shown that, in addition, this leads to PMNL migration across endothelial cell monolayers grown either on solid surfaces or on microporous filters.[5,8] This effect appears, at least in part, to be due to the induction of adhesion molecules for PMNL on the surface of the endothelium.[4,6,9]

Once PMNL have traversed the vascular wall, in most tissues they migrate into connective tissue, which is rich in fibroblasts or fibroblast-like cells. Thus, there may be additional mechanisms in the extravascular space involved in the migration of PMNL, which may contribute to the tissue infiltration by PMNL accompanying inflammatory reactions. IL-1 and TNF-α have both been found in inflammatory reactions and, in addition to effects on vascular endothelium, these cytokines have a plethora of biological actions on a large variety of cells. They have profound effects on connective tissue fibroblasts by influencing their proliferation, prostaglandin production, collagenase secretion, expression of cell-surface molecules, and the secretion of other cytokines such as IL-6, IL-8 and colony-stimulating factors (reviewed in refs 10–15). We have therefore investigated the effects of IL-1 and TNF-α on PMNL interaction with fibroblasts and contrasted this with our previous work on PMNL interaction with cytokine-stimulated endothelium. Our findings indicate that, although IL-1 and TNF-α induce similar biological effects in promoting PMNL adhesion

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and migration across vascular endothelium, TNF-α selectively activates fibroblasts in a manner which promotes PMNL migration across confluent fibroblast monolayers. These findings may be relevant to the migration of PMNL through the extravascular connective tissue during inflammation.

MATERIALS AND METHODS

Growth of endothelial cells and fibroblasts

Human umbilical vein endothelial cells (HUVE) were isolated and cultured as described by Jaffe *et al.*[16] using modifications as described previously.[5] Briefly, endothelial cells were isolated from umbilical cords after treatment with 0·5 mg/ml collagenase (Cooper Biomedical, Mississauga, Ontario, Canada), in 0·01 M phosphate-buffered saline (PBS), pH 7·4, and grown in RPMI-1640 containing 2 mM L-glutamine, 2-mercaptoethanol (2-ME), sodium pyruvate, penicillin G/streptomycin (Gibco, Grand Island, NY) and supplemented with 20% foetal calf serum (FCS; Hyclone, Logan, UT), 50 μg/ml endothelial cell growth supplement (Collaborative Research, Bedford, MA) and heparin (90 μg/ml; Sigma Chemical Co., St Louis, MO). Cells were cultured in 25-cm² culture flasks (Nunc, Gibco), which were previously coated with 2% gelatin (Difco, Detroit, MI). The cells had the typical cobblestone morphology and >98% showed staining for factor VIII antigen by immunofluorescence. At confluence HUVE were detached using 0·025% trypsin/0·01% Versene (MA Bioproducts, Walkerville, MD) and cultured on PVP-free polycarbonate filters bearing 3 μm pores in Transwell culture plate inserts (6·5 mm diameter, Transwell 3415; Costar, Cambridge, MA). The filters were prepared by coating with 30 μl of 2% gelatin for 30 min followed by removal of excess fluid and air drying. Then 3 μg of human fibronectin (Collaborative Res.) in 30 μl water were allowed to dry onto the filter. HUVE at 2×10^4 cells, from the first or second passage, were added to the cups above the filter in 0·1 ml complete medium and 0·6 ml medium was added to the lower compartment beneath the filter. The HUVE formed a tight permeability barrier in 6–7 days. Medium was exchanged for fresh medium 2 days before (usually Day 4 or 5) the monolayers were used for migration studies on Day 6 or 7.

Human dermal fibroblasts were isolated from foreskin obtained from newborn babies or from skin biopsies. The minced tissue was cultured in RPMI-1640, 20% FCS, penicillin/streptomycin, until fibroblasts grew out of the tissue. These were serially passaged until cultures were homogeneous. Some of the fibroblasts were donated to us from the Cytogenics Laboratory, the I.W.K. Children's Hospital (Halifax, NS) after confirmation of normal karyotype. Cells between the third and tenth passage number were used. They were grown to confluence in culture flasks using RPMI-1640, 20% FCS, 2-ME and antibiotics, harvested with trypsin 0·05%/EDTA 0·02% (Flow laboratories, Mississauga, Ontario, Canada) and seeded onto gelatin-coated Transwell filters, as was the case for the HUVE, at 2×10^4 cells in 0·1 ml. Fibronectin coating was not required for fibroblast attachment. Fibroblasts on the filters were cultured in RPMI-1640 with 20% FCS, 2-ME and antibiotics for 6–7 days, as described above.

In some experiments, fibroblasts were cultured in 2-cm² or 9-cm² gelatin-coated tissue culture wells, using the same conditions as above, until they achieved confluence, when the medium was removed and the wells were washed and fed with RPMI-1640, 5% FCS. These cells were either stimulated or unstimulated for 4–20 hr. The culture supernatants were then harvested, centrifuged to remove cells, and stored frozen at −70° until testing.

Test of monolayer permeability and histology

To evaluate the functional integrity of the HUVE monolayer, 30 μl of ^{125}I-labelled human serum albumin (HSA; Connaught Labs., Toronto, Ontario, Canada) in RPMI-1640, 10% FCS were added to the 100 μl medium in the upper compartment above the HUVE monolayer on the filters. Forty-five minutes later, a 30-μl sample was taken from the lower chamber beneath the filter for ^{125}I determination, using an LKB 1240 Gamma Counter. The percentage of total counts added to the upper chamber present in the total volume in the lower chamber was calculated using the formula:

$$\% \text{ diffusion} = \frac{\text{total c.p.m. in lower chamber}}{\text{total c.p.m. added to upper chamber}} \times 100.$$

Histological preparation of filters was kindly performed by Ms M. Henry (I.W.K. Hospital, Halifax, NS, Canada). The monolayer-filter unit was fixed with 2% glutaraldehyde, post-fixed in OsO_4, dehydrated in ethanol and acetone and embedded in plastic by standard techniques. One-micron thin sections were stained with toluidine blue.

Preparation of PMNL suspension and PMNL migration

Peripheral venous blood from healthy human individuals was collected into heparin (5 U/ml blood), acid citrate dextrose (ACD formula A) and red cells were separated by sedimentation with 6% dextran saline solution (Travenol, Malton, Ontario) (1 part to 5 parts blood). The leucocyte-rich plasma was harvested. Leucocytes and residual red cells were centrifuged (150 ***g***, 10 min) into a pellet and resuspended in Ca^{2+}, Mg^{2+}-free Tyrode's solution with 5% autologous platelet-poor plasma (PPP) and labelled with ^{51}Cr sodium chromate (50 μCi/ml; Amersham Corp, Oakville, Ontario, Canada). Labelled PMNL were separated by centrifugation (300 ***g***, 30 min) on discontinuous (62%/73%) 10% PPP-Percoll (Pharmacia Fine Chemicals, Dorval, Quebec, Canada) gradients. After two washes with the Tyrode's solution containing 0·5% HSA, the PMNL were resuspended at a final concentration of 3×10^6/ml in RPMI-1640, 0·5% HSA containing 10 mM HEPES. This method yielded >95% pure PMNL with essentially no red cell contamination.

For assays, HUVE or fibroblast monolayers on the filters and the lower compartment were washed with RPMI-1640 and stimulated, usually for 3 hr, by addition of TNF-α or IL-1α to the lower compartment in fresh RPMI-1640 with 10% FCS and 10 mM HEPES. Recombinant IL-1α was a gift from Dr P. Lomedico (Hoffman La Roche, Nutley, NJ). It had a specific activity of $2{\cdot}1 \times 10^7$ U/mg and contained <20 ng endotoxin/mg. Recombinant TNF-α was a gift from Dr M. Shepard (Genentech Inc., San Francisco, CA). This preparation had a specific activity of 5×10^7 U/mg and contained <0·1 ng endotoxin/mg. The cytokines were diluted in PBS containing 0·1% pyrogen-free HSA. Following preincubation with cytokines, the upper and lower surfaces of the filter cups were washed with RPMI-1640 and then they were transferred to a new, clean well (lower compartment). To this well, 0·6 ml of RPMI-1640, HEPES, 0·5% HSA was added and, before immersion of the filter cup,

0·1 ml of [^{51}Cr]PMNL suspended in the same medium was added to the upper compartment. After various incubation times (30–120 min), migration was stopped by vigorous washing of the upper compartment twice with 0·1 ml of RPMI-1640 to remove non-adherent PMNL. The undersurface of the filter was then rinsed with 2 ml of ice-cold PBS/0·2% EDTA solution and collected into the lower compartment. The filter cup (upper compartment) was then placed into 0·7 ml of 0·5 M NaOH to allow dissolution of adhered PMNL. The PMNL which migrated into the lower compartment or were detached from the filter were lysed by addition of 0·5% Triton X-100. The NaOH solution bathing the filters (adherent PMNL-^{51}Cr) and the contents of the lower chamber (medium + PBS EDTA wash of filter undersurface) (migrated PMNL) were analysed for ^{51}Cr and results are expressed as the percentage of total [^{51}Cr] PMNL in each fraction.

Migration of PMNL across bare filters was measured using ^{51}Cr-labelled PMNL or by Coulter particle counting of PMNL in the lower chamber, as described previously.[17] In these assays, the PMNL were also suspended in RPMI-1640, 0·5% HSA, 10 mM HEPES, but incubation time at 37° for PMNL migration was only 40 min, because longer incubations resulted in excess random (unstimulated) transfilter migration. Only the PMNL in the lower chamber plus those cells which washed off the filter undersurface were counted as 'migrated'. The positive chemotactic stimulus, zymosan activated plasma (ZAP), as a source of C5a des Arg was generated and used as previously (3), by addition to the lower compartment at the time of PMNL addition to the upper chamber. All stimulation conditions were performed with triplicate replicates.

Monoclonal antibody treatments

In some experiments, ^{51}Cr-PMNL were treated for 30 min at room temperature with one of the following monoclonal antibodies, and then washed prior to migration assays. The monoclonal antibodies (mAb) employed were 60.3, which recognizes a functional epitope on CD18 (kindly provided by Dr J. Harlan, University of Washington, Seattle, WA),[18] mAb TS 1/22 and LM 2/1, which react with CD11a and CD11b, respectively (kindly provided by Dr T. Springer, Harvard Medical School, Boston, MA),[19] control mAb W 6/32 (American Tissues Culture Collection, Rockville, MD), which reacts with HLA class I framework, and mAb 3H8, which reacts with pertussis toxin (gift from Dr S. Halperin, Dalhousie University, Halifax, NS). The antibodies were used at 40 μg/ml of purified IgG (60.3), or as ascites at 1:200 for TS 1/22, LM 2/1, 3H8 and W 6/32. Rabbit antiserum to human IL-8 (kind gift from R. Marks, University of Michigan, Ann Arbor, MI) was used at 1:100 dilution for 2 hr to neutralize IL-8.

Enzyme-linked immunoassay on fibroblasts

The fibroblasts on filters or in microwells were stimulated for 3 hr with cytokines at the indicated concentrations. Expression of surface molecules was assayed as described previously.[5] Briefly, the stimulants were removed and replaced by 100 μl RPMI-1640, 5% FCS, 0·1% NaN_3 containing mAb W 6/32 (ATCC) or mAb RR1/1 anti-ICAM-1 (gift from Dr T. Springer) at 1:1000 of ascites, or mAb H4/18 anti-ELAM-1 (gift from Dr M. Gimbrone, Brigham and Women's Hospital, Boston, MA) at 1:1000 of ammonium sulphate-precipitated γ-globulin from ascites, or mAb 3H8 anti-pertussis toxin (negative control) at

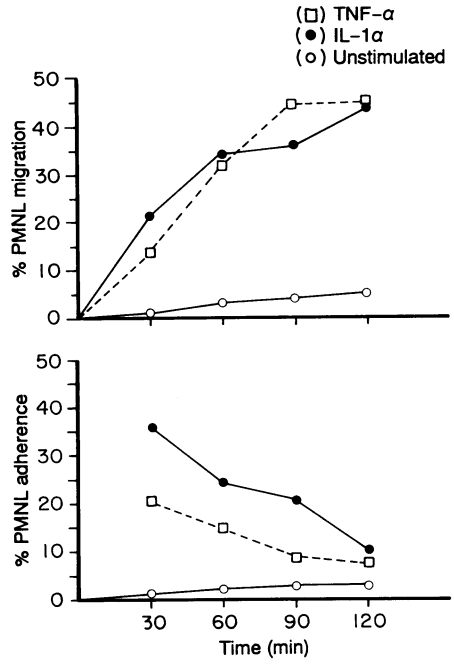

Figure 1. Effect of TNF-α and IL-1α on PMNL adhesion to and migration through endothelium. Umbilical vein endothelial monolayers grown on polycarbonate filters were stimulated with TNF-α (10 U/ml) or IL-1α (2 U/ml) for 3 hr, medium was then exchanged and ^{51}Cr-labelled PMNL were added above the monolayer. The system was incubated for durations up to 120 min, during which PMNL adhesion and migration occurred. These parameters were quantified as described in the Materials and Methods. One representative experiment of six time-course experiments performed in triplicate is shown.

1:1000 of ascites. After 60 min incubation (37°, 5% CO_2) the monolayers were washed twice with RPMI-1640, 5% FCS, 0·1% azide and twice with RPMI-1640. One-hundred microliters of peroxidase conjugated goat anti-mouse IgG (Cappell Labs, Malvern, PA), diluted 1:3000 in RPMI-1640, 5% FCS, were added to the monolayers and incubated for 1 hr. The monolayers were then washed twice with PBS, 5% FCS, then in PBS, and finally in 0·9% NaCl. One-hundred microtitres of O-phenylenediamine 12·5 mg/ml (Sigma Chemical Co.) in 0·1 M citrate-phosphate buffer, pH 5, containing 0·012% H_2O_2 were then added and after 15–20 min the enzyme reaction was stopped by adding 100 μl of 4·5 M H_2SO_4. The absorbance was read at 492 nm.

Statistical methods

Values shown are means and standard error of the means. Student's *t*-test was used in calculating *P* values.

RESULTS

Initial experiments confirmed that HUVE formed a morphologically confluent monolayer on the filters,[5] as reported previously. These monolayers formed a tight permeability barrier through which less than 1% of ^{125}I-HSA diffused in 45 min (control >20%). Figure 1 shows the effect of IL-1α or TNF-α treatment of HUVE for 2–3 hr on subsequent PMNL interactions. In these experiments IL-1 or TNF-α was removed by washing and medium exchange prior to the addition of PMNL.

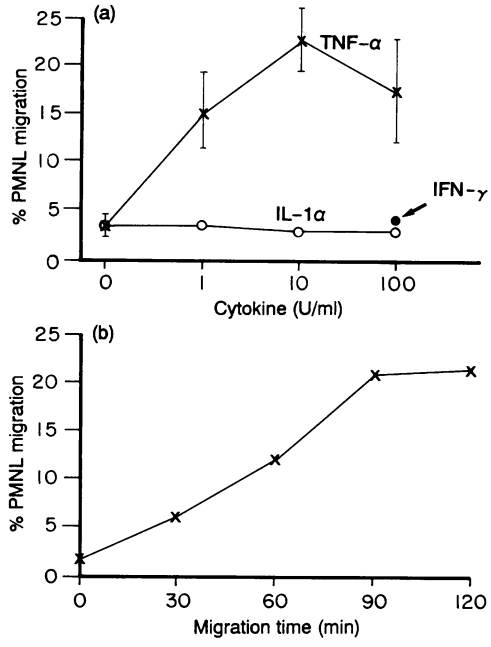

Figure 2. The migration of PMNL across fibroblast monolayers stimulated with TNF-α, IL-1α and IFN-γ. Fibroblast monolayers grown on polycarbonate filters were stimulated with the indicated concentration of cytokines followed by washing and medium exchange. Labelled PMNL were then added and PMNL migration through the fibroblasts and filter were quantified as in Fig. 1 after 90 min incubation (a). In the time-course experiments (b), 10 U/ml TNF-α were the stimulant. Medium containing TNF-α (10 U/ml) did not induce PMNL migration in the absence of fibroblasts. Values in (a) are means ± SEM of at least four dose-response experiments; (b) is a representative experiment of three performed with triplicate replicates.

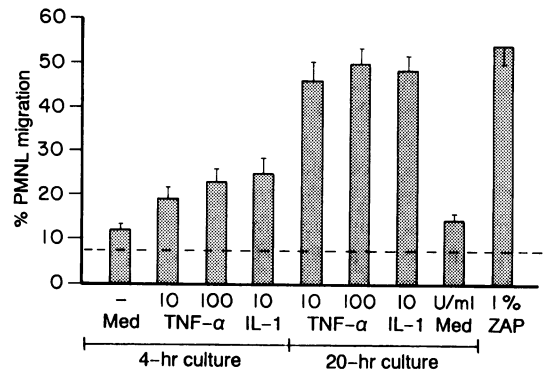

Figure 3. The effect of fibroblast culture supernatants on PMNL migration. Confluent fibroblast monolayers were stimulated for 4 or 20 hr under various conditions and the culture supernatants were harvested. Supernatants were added at a 1:10 dilution to the lower compartment and PMNL migration across bare filters was measured. Broken line shows activity of medium (Med) ± cytokine alone (no fibroblasts).

Under these conditions, a significant proportion of the PMNL adhered to the monolayer within the first 30 min, whereas there was virtually no adherence of PMNL to unstimulated HUVE. PMNL migration across the endothelial and filter unit into the lower chamber was already detectable at 30 min, and progressively increased for 60–90 min with nearly half of the PMNL traversing the monolayer and filter by 90 min. There was virtually no migration across monolayers that were not stimulated (≤ 4). As the time of incubation increased, there was a consistent decline in the degree of PMNL adhesion to these monolayers, perhaps as a result of transendothelial migration. Although similar experiments have been reported by us previously,[5] these results are included here for comparison with results obtained when human dermal fibroblasts were cultured on the filters.

After 6–7 days of culture, the fibroblasts also formed a confluent monolayer and a permeability barrier to the diffusion of ^{125}I-HSA, although this was not as tight a barrier as was the case with the endothelial monolayers. In the case of fibroblasts, monolayer permeability to albumin in 45 min averaged 4%, whereas that of HUVE monolayers was $< 1\%$, and that of bare filters was $> 20\%$. As for endothelial cells, fibroblast monolayers were incubated with medium containing various concentrations of TNF-α or IL-1α, as shown in Fig. 2. Preincubation for 3 hr with human TNF-α induced PMNL transfibroblast migration, even when the TNF-α was removed from the system by washing and medium exchange. Optimal migration was observed with approximately 10 U/ml of TNF-α. In contrast, IL-1α treatment of the fibroblast layer did not induce PMNL migration over a wide concentration range. In these experiments, pretreatment of the fibroblasts for 2–4 hr was found to be optimal for inducing subsequent PMNL migration. None of the cytokines induced PMNL migration across filters in the absence of fibroblasts. Because IFN-γ is also known to exert effects on fibroblasts[10,12] its effects were also studied. Treatment of the fibroblasts for either 3 or 24 hr with IFN-γ did not induce PMNL migration across the monolayer.

Figure 2b shows the time-course of PMNL migration across TNF-α-stimulated fibroblasts. The kinetics of PMNL migration were similar to those observed for PMNL migration across HUVE monolayers grown on the membrane filters, i.e. progressive migration over a 90 min period to a plateau. Presumably, the migration is preceded by a brief period of PMNL adhesion to the fibroblast layer. However, in contrast to the HUVE monolayers, on which TNF-α or IL-1 treatment induced adhesion of 20–35% of the PMNL (unstimulated = 2–3%) within the first 30 min, in the case of the TNF-α-activated fibroblasts, PMNL adhesion to the monolayer was far less even at 30 min, when PMNL migration was just beginning. With fibroblasts, PMNL adhesion to unstimulated monolayers at 30 min was $1{\cdot}8 \pm 0{\cdot}2\%$ of added cells, compared with $3{\cdot}5 \pm 0{\cdot}4\%$ on TNF-α-stimulated monolayers.

Fibroblasts have been shown to produce molecules chemotactic for PMNL, which can induce PMNL migration *in vitro*.[10,14] We therefore considered that the TNF-α-activated fibroblasts might induce PMNL migration across the monolayers via such a secreted factor. To investigate this, fibroblasts were grown to confluence in macrowell plates, and then stimulated for 4 or 20 hr with TNF-α or IL-1. The culture supernatants were tested for activity inducing PMNL migration across the bare filters. As shown by the broken line in Fig. 3, medium alone, with or without the cytokines (IL-1α, TNF-α) in the *absence* of fibroblasts, induced migration of 8% of the PMNL. Supernatants from fibroblast cultures under unstimulated conditions induced slightly more PMNL migration than medium by itself (12–15%). However, stimulation of the fibroblasts with TNF-α 10–100 U/ml or IL-1α 10 U/ml for 4 hr

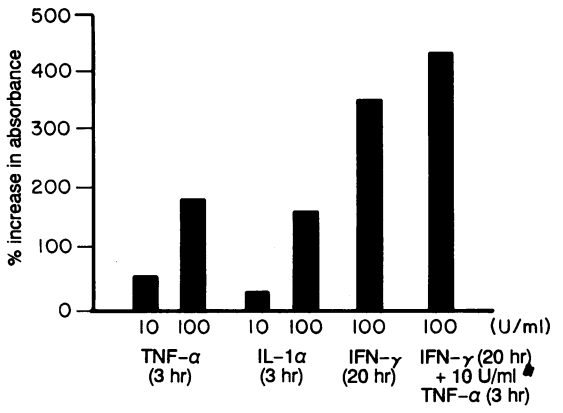

Figure 4. The effect of cytokines on ICAM-1 expression by fibroblasts. Expression of ICAM-1 on viable fibroblast monolayers was measured by ELISA as described in the Materials and Methods. One representative experiment of three is shown. Values are means of triplicate replicates where absorbances differed ≤15%. Background absorbance was 0·08 and with unstimulated fibroblasts was 0·210 OD.

Table 1. Effect of cycloheximide and antibody to IL-8 on PMNL transfibroblast migration

Treatment	% inhibition of PMNL migration with TNF-α-stimulated* fibroblasts	% inhibition of PMNL migration with ZAP† + fibroblasts
Cycloheximide (10 μg/ml)	93±4%	5±3%
Anti-IL-8	0±10%	0±4%

*Cycloheximide was added to fibroblasts at time of TNF-α stimulation and also during 90 min incubation with PMNL. Antiserum to IL-8 was added (1:200 final) to PMNL suspension just before addition above fibroblast layer.

†Zymosan-activated plasma (ZAP, C5a des Arg) at 1% final was added to the lower compartment beneath fibroblast and filter unit, immediately before addition of PMNL, in order to induce PMNL migration via a non-fibroblast-dependent mechanism. Values are means±SEM of three experiments performed in triplicate.

or 20 hr resulted in accumulation of activity in the culture supernatants which induced PMNL migration. After 20 hr of culture with TNF-α or IL-1, the fibroblast supernatants induced as much PMNL migration as the maximal response elicited by 1% ZAP, shown by the bar on the far right as a reference positive control. The results of these experiments contrast with observations using the fibroblast monolayers, where IL-1 did not activate for induction of PMNL transfibroblast migration.

Recently, it has been reported that the major PMNL chemotactic factor produced by human dermal fibroblasts in response to IL-1 or TNF-α is IL-8 (NAP-1).[14] In agreement with this, the chemotactic activity in TNF-α and IL-1α-stimulated culture supernatants, shown in Fig. 4, was inhibited by 80% by anti-IL-8 serum (not shown). We therefore investigated the potential role of IL-8 production by the fibroblast monolayers on the filters in the TNF-α-induced PMNL transfibroblast migration. Following activation of the fibroblasts by TNF-α for 3 hr, the medium was exchanged and neutralizing rabbit anti-human IL-8 antiserum or non-immune rabbit serum (control) was added at the time of PMNL addition, to neutralize any continued endogenous IL-8 production by the fibroblasts during the incubation with PMNL. The amount of anti-IL-8 serum was sufficient to completely neutralize at least 50 ng/ml of IL-8. As can be seen from Table 1, it did not affect the migration of PMNL across TNF-α-stimulated fibroblasts. We also examined the requirement for protein synthesis by the fibroblasts during the TNF-α stimulation period (3 hr) for induction of PMNL transfibroblast migration. The protein synthesis inhibitor, cycloheximide, added to the fibroblast monolayers during the TNF-α activation stage, nearly completely inhibited (93%) the ability of the fibroblast monolayer to support PMNL transmigration. Cycloheximide treatment of the fibroblasts and/or the PMNL, at the dose used, did not inhibit PMNL transfibroblast and transfilter migration induced by ZAP, when this stimulus was added to the lower chamber. The fibroblast monolayer remained intact as assessed by permeability, at the end of the cycloheximide treatment. Thus the synthesis of protein by the fibroblasts appears necessary for TNF-α activation to support PMNL transfibroblast migration.

PMNL interaction with vascular endothelium is now recognized to be in part due to the expression on 'activated' endothelium of surface adhesion molecules, with which PMNL and other leucocytes can interact and of which the two best defined are intercellular adhesion molecule-1 (ICAM-1) and endothelial leucocyte adhesion molecule-1 (ELAM-1).[4,9,20] Using HUVE grown on the Transwell culture insert membranes, we have observed that IL-1α and TNF-α strongly upregulate the surface expression of these molecules, as measured by ELISA on viable monolayers.[5] Applying the same methods to fibroblasts, we observed no expression of ELAM-1 under resting or IL-1α- or TNF-α-stimulated conditions (not shown). However, fibroblasts grown on the filter system did express low levels of surface ICAM-1 (0·210 absorbance units) (not shown). Furthermore, as shown in Fig. 4, 3-hr stimulation of the fibroblast monolayers with TNF-α or IL-1α increased the surface expression of ICAM-1 to the same degree, by up to 180%, as measured by increase in absorbance. IFN-γ treatment for 20 hr, or combination treatment with IFN-γ and TNF-α, increased it even more. Thus, while all the cytokines upregulated ICAM-1 expression, only TNF-α also induced PMNL transfibroblast migration (Fig. 2).

Finally, we also determined which adhesion molecules are required for PMNL migration across the fibroblasts. The CD11/CD18 glycoprotein complex, belonging to the β_2 integrin family, are well-characterized adhesion molecules on PMNL, which are required for adhesion and PMNL migration *in vitro* and *in vivo* (reviewed in refs 4, 6, 19). We therefore investigated their involvement in the PMNL migration response (Table 2). The ^{51}Cr-labelled PMNL were treated with saturating concentrations of mAb TS 1/22 (anti-CD11a) or LM 2/1 (anti-CD11b) or mAb 60.3 (anti-CD18) or control mAb W 6/32 (anti-HLA class I framework) or 3H8 (anti-pertussis toxin). The PMNL were treated for 30 min, washed, and then added to the upper compartment of the migration system above the TNF-α-activated fibroblast monolayer. While anti-CD18 antibody blocked PMNL migration virtually completely, treatment with anti-CD11a or anti-CD11b was nearly as effective, inhibiting migration by about 80%. Treatment of the PMNL with the

Table 2. Effect of CD11/CD18 antibody on PMNL transfibroblast migration

	Anti-CD11a* (LFA-1)	Anti-CD11b (MAC-1)	Anti-CD18	Anti-HLA class I
% inhibition	82±8	78±9	90±5	5±3

*The ^{51}Cr-labelled PMNL were treated with mAb TS 1/22 (anti-CD11a), LM 2/1 (anti-CD11b), 60.3 (anti-CD18) or W 6/32 (anti-class I) as indicated in the Materials and Methods, for 30 min at 22°, and then washed prior to addition above TNF-α (10 U/ml)-stimulated fibroblast monolayers. Migration was for 90 min. Values are means ± SEM of at least three experiments performed in triplicate.

control HLA class I antibody, W 6/32, did not significantly inhibit PMNL migration, although the amount of antibody binding to the PMNL was comparable to that of the other antibodies.

DISCUSSION

Our findings indicate that TNF-α can stimulate fibroblasts to facilitate PMNL migration across a fibroblast barrier. This effect is fibroblast dependent, and the continued presence of TNF-α is not required. The fibroblast activation was time-dependent, being optimal after 2–4 hr of TNF-α exposure, and required protein synthesis by the fibroblasts, since cycloheximide blocked the response (Table 1). Although IL-1 and TNF-α are known to exert many similar biological effects on fibroblasts and to induce some of the same mRNA transcripts,[10,12-15,21] IL-1α did not share the above action of TNF-α in our system. This was in marked contrast to the similar effects of IL-1α and TNF-α in activation of endothelium for PMNL adhesion and transendothelial migration (Fig. 1). Furthermore, IL-1α did induce other responses, e.g. chemotactic factor production (Fig. 3), confirming that the fibroblasts employed were responsive to IL-1.

The mechanisms involved in PMNL transfibroblast migration appear to differ from those involved in PMNL transendothelial migration. The latter is characterized by strong adhesion of PMNL to IL-1- or TNF-α-stimulated endothelium, reaching 10–20-fold more than to unstimulated endothelium (Fig. 1). This adhesion is likely to be due to endothelial expression of ELAM-1 and ICAM-1.[4,6,9] In contrast, the PMNL adhesion to TNF-α-stimulated fibroblasts was only slightly increased over unstimulated fibroblasts, by 2–3-fold, equivalent to 3–4% of PMNL, in spite of good transfibroblast migration. It is possible that under the washing conditions employed, which were designed to minimize the PMNL binding to unstimulated monolayers, a weak adhesive interaction between PMNL and TNF-α-activated fibroblasts may be obscured. On the other hand, the PMNL binding to IL-1- or TNF-α-activated endothelium appears to be exceptionally strong. Several other factors, e.g. C5a, LTB_4, formyl peptides, which are potent inducers of PMNL migration, cause weaker and much more transient adhesion to endothelium than do IL-1 or TNF-α, at least *in vitro*.[22,23]

An analysis of the adhesion molecules ELAM-1 and ICAM-1 revealed that fibroblasts did not express ELAM-1, but ICAM-1 was basally expressed and IL-1α, TNF-α, and IFN-γ all up-regulated its expression. However, this response alone cannot account for the effect of TNF-α on the fibroblast–PMNL interaction, since neither of the other cytokines induced fibroblasts to support PMNL transmigration. Thus, additional mechanisms or surface molecules may be selectively stimulated by TNF-α on fibroblasts to account for the observed PMNL migration.

Both IL-1 and TNF-α are known to induce fibroblasts to produce chemotactic factors, in particular IL-8 (NAP-1).[14] The chemotactic activity we detected in IL-1α- or TNF-α-stimulated fibroblast culture supernatants (Fig. 3) was >80% neutralized by monospecific anti-IL-8 serum, confirming the above reports. However, the mechanism whereby TNF-α induces PMNL transfibroblast migration does not appear to depend on IL-8 production because: (i) IL-1 and TNF-α were equally active in inducing production of chemotactic activity by fibroblasts for induction of PMNL transfibroblast migration; and (ii) antiserum to IL-8 did not inhibit PMNL transfibroblast migration following TNF-α activation of the fibroblast layer. Thus, if a soluble factor is involved, it seems to be distinct from IL-8 (NAP-1).

Our results suggest that whatever the mechanism involved, the PMNL migration response is highly dependent on the function of the PMNL (CD11/CD18) surface molecules. Both CD11a (LFA-1) and CD11b (MAC-1) appear to be involved, since blocking either of them on PMNL nearly completely (≈80%) inhibited transfibroblast migration (Table 2). This finding is more striking than that seen with PMNL-endothelial interactions, in which blocking of CD11a or CD11b selectively usually results in only partial (30–50%) inhibition of adhesion or migration.[6,22,23]

In summary, we may have found a novel interaction between PMNL and connective tissue fibroblasts exposed to TNF-α. It is possible that such an effect of TNF-α may be relevant to our previous observation and that of Movat *et al.*[2,3] that TNF-α could markedly and synergistically increase the accumulation of PMNL in dermal inflammation induced by IL-1. Those findings suggested that IL-1 and TNF-α may exert some pro-inflammatory actions through distinct mechanisms, which may complement each other. The ability of TNF-α, but not IL-1α, to stimulate fibroblasts to facilitate PMNL migration, e.g. once PMNL reach the extravascular space, may be one example of this and may account for the synergy observed *in vivo* in tissue PMNL accumulation. Since such synergistic pro-inflammatory actions of IL-1 and TNF-α are of potential detrimental effect in certain disease processes, further investigation of TNF-α effects on connective tissue cell–PMNL interactions appears warranted.

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