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Polymorphonuclear leucocyte migration through human dermal fibroblast monolayers is dependent on both β_2-integrin (CD11/CD18) and β_1-integrin (CD29) mechanisms

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SUMMARY

Accumulation of leucocytes in inflammation involves their migration through vascular endothelium and then in the connective tissue. We investigated human polymorphonuclear leucocyte (PMNL) migration through a biological barrier of human dermal fibroblasts grown on microporous filters, as a model of PMNL migration in the connective tissue. PMNL did not migrate through a fibroblast monolayer unless a chemotactic factor, e.g. C5a, interleukin-8 (IL-8) or zymosan-activated plasma (ZAP; $C5a_{desArg}$), was added. This migration was partially inhibited (35–70%, depending on the stimulus) by treatment of PMNL with monoclonal antibody (mAb) to CD18 (β_2-integrins). Most of the CD18-independent migration was inhibited by mAb to β_1-integrins (CD29). Inhibition by mAb to β_1 was observed when the PMNL, but not the fibroblasts, were treated with mAb. The role of β_1-integrins in PMNL transfibroblast migration was detectable only when the function of the CD11–CD18 complex was blocked, because mAb to β_1-integrin alone had no significant effect on PMNL migration. Migration induced by C5a was more CD18-independent compared to IL-8 or $C5a_{desArg}$. The CD18-independent migration was also inhibited by mAb to the β_1-integrin subunits α_5 (of very late antigens-5; VLA-5) and α_6 (of VLA-6). Treatment of the fibroblasts (4 hr) with tumour necrosis factor-α (TNF-α) or IL-1α enhanced C5a-induced PMNL transfibroblast migration and increased the proportion of migration utilizing the CD11–CD18 mechanism. However, TNF-α treatment had no effect on the degree of β_1-integrin-dependent migration. These findings suggest that in response to the chemotactic factors C5a, IL-8 and $C5a_{desArg}$, PMNL migration in the connective tissue is mediated by both CD11–CD18 (β_2) and β_1-integrins on the PMNL. The VLA-5 and VLA-6 members of β_1-integrins are involved in this process. This is in contrast to PMNL migration across endothelium in this system, which is virtually all CD18 dependent with no significant role for β_1-integrins.

INTRODUCTION

Leucocyte infiltration into tissues is a prominent feature of inflammation. The mechanism of leucocyte migration across blood vessel endothelial cells have been investigated extensively and reviewed recently.[1–3] It is recognized that at least three steps are required for polymorphonuclear leucocyte (PMNL) migration, i.e. rolling of PMNL on the vascular endothelium via selectin–carbohydrate receptor–ligand interactions, activation of the leucocyte by chemokines and other chemotactic factors (e.g. platelet-activating factor; PAF), probably present on the surface of the endothelial cells, and firm adhesion via β_2-integrin (CD11–CD18) receptors on the PMNL to endothelial cells expressing appropriate counter-receptors or cell adhesion molecules (CAM), i.e. intercellular adhesion molecule-1 (ICAM-1) and 2.[1–3] This is followed rapidly by migration through endothelial gap junctions, which probably involves platelet endothelial cell adhesion molecule (PECAM-1).[4] Subsequently, PMNL must move through the subendothelial basement membrane and towards inflammatory stimuli through connective tissue composed of stromal cells, e.g. fibroblasts and extracellular matrix (ECM) proteins such as fibronectin, laminin, vitronectin, collagen, etc.[5] It is probable that PMNL migration through connective tissue also involves interaction between adhesion molecules on the leucocytes, connective tissue cells and ligands in the ECM. The β_2 (CD18) and β_1 (CD29) integrin family members on PMNL have been shown to mediate adhesion to ECM proteins such as laminin, and fibronectin, as well as to denatured proteins.[6–10]

The β_1-integrins consist of individual α-chains and a common β_1-chain (CD29) and there are at least nine heterodimer members.[11,12] Most of the members on a variety of leucocytes and connective tissue cells are receptors for ECM

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proteins such as fibronectin, laminin and collagen.[6,11,12] The adhesion of PMNL to laminin or fibronectin was found to be partially inhibited by monoclonal antibody (mAb) to the β_1-integrin chain.[6,9] Furthermore, one PMNL receptor for laminin has been identified as very late antigen-6 (VLA-6; $\alpha_6\beta_1$),[9,13] and one for fibronectin as VLA-5 ($\alpha_5\beta_1$).[6] Most of these studies employed *in vitro* adhesion assay systems in which ECM proteins were immobilized on a non-biological surface.

In contrast to PMNL adhesion to ECM proteins, little is known about the molecular mechanisms involved in leucocyte migration in the connective tissue in the presence of connective tissue cells. The best characterized integrins on PMNL with a clear role in migration are the β_2-integrins (CD11–CD18). The β_2-integrins are present only on leucocytes and consist of three heterodimeric subunits, each with a separate α-chain ($CD11_{a-c}$) and a common β_2-chain (CD18). These heterodimers are designated leucocyte function-associated antigen-1 (LFA-1) (CD11a/CD18), Mac-1 or CR3 (CD11b–CD18), and p150,95 (CD11c–CD18).[1–3] The β_2-integrins in part mediate PMNL adhesion to vascular endothelium, and are essential for PMNL transendothelial migration.[1,2,14,15] They also appear to have a role in PMNL chemotaxis on ECM protein-coated surfaces.[8] Previously, we have employed a polycarbonate filter system (Transwell) on which we cultured human dermal fibroblasts (HDF) to form a biological barrier, and studied the migration of PMNL through this surface.[16] With this system we found that PMNL migrated across a dermal fibroblast monolayer if the fibroblasts were stimulated by tumour necrosis factor-α (TNF-α), and this migration was completely dependent on the CD11–CD18 integrin complex on the PMNL.[16] Here we used this system to investigate the molecular mechanisms of PMNL migration through such a connective tissue barrier when migration is induced by a chemotactic factor, as is likely to be the case in inflammatory reactions. We observed that PMNL migrate across a fibroblast barrier rapidly in response to chemotactic factors. This migration is only partly CD18 dependent and there is also an alternative pathway mediated largely by β_1-integrins, including the VLA-5 and VLA-6 integrins on the PMNL.

MATERIALS AND METHODS

Monoclonal antibodies

The following murine (except where indicated) mAb against human antigens were used as purified IgG: 60.3 (anti-CD18; IgG2a; provided by Bristol-Myers Squibb, Seattle, WA);[17] R15.7 (anti-CD18; IgG1; provided by Dr R. Rothlein, Boehringer Ingelheim, Ridgefield, CT); rat mAb 16 (anti-α_5 subunit of the VLA-5; IgG2a); and mAb13 (anti-β_1 integrin; IgG_1) were provided by Dr K. Yamada National Institute of Health, Bethesda, MD);[18] and BB11 (anti-E-selectin; IgG2b; from Dr R. Lobb, Biogen Inc., Cambridge, MA).[19] Two new murine mAb, 3S3 and 6S8, reactive with human β_1-integrin, were provided as purified IgG by Dr J. Wilkins (University of Manitoba, Winnipeg, MB). These mAb react specifically with CHO cells transfected with the full-length human β_1-integrin gene and with affinity-purified β_1-integrin by enzyme-linked immunosorbent assay (ELISA; J. A. Wilkins, N. Hunt, D. G. Stupack, H. Ni and C. Shen, manuscript submitted for publication). Monoclonal antibodies 3S3 and 6S8 also precleared all β_1 reactivity for the anti-β_1 mAb JB1[20] and inhibited $\alpha_4\beta_1$-integrin-dependent lymphocyte adherence to fibronectin (J. A. Wilkins *et al.*, unpublished observations). The following mAb were used as ascites: 135-13C (rat mAb) and 450-30A1 (anti-α_6-chain of VLA-6; IgG1; from Dr S. J. Kennel, Oak Ridge National Laboratory, TN);[21] 3H11B9 (anti-pertussis toxin; IgG1; from Dr S. Halperin, Halifax, NS, Canada); 543 (anti-CR1; IgG1) and 3C10 (anti-CD14; IgG1; from ATCC Bethesda, MD). Culture supernatant of rat mAb GoH3 against the α_6 subunit of VLA-6 (IgM) was a gift from Dr A. Sonnenberg (the Netherlands Cancer Institute, Amsterdam, the Netherlands).

Reagents

Recombinant human TNF-α (specific activity 5×10^7 U/mg) was a gift from Genentech Inc. (South San Francisco, CA). Recombinant human interleukin-1α (IL-1α; specific activity 4×10^7 U/mg) was a gift from Dr D. Urdal (Immunex Corp., Seattle, WA). Recombinant human IL-8 (NAP-1) was a gift from Sandoz Pharmaceutical (Vienna, Austria). These cytokines contained < 1 ng lipopolysaccharide (LPS))/mg and were diluted immediately before use in 0·1% LPS-free serum albumin (HSA; Connaught Laboratories, Don Mills, Ontario, Canada) in phosphate-buffered saline PBS. Recombinant human C5a was a gift from CIBA-Geigy Pharmaceuticals (Summit, NJ). Human zymosan-activated plasma (ZAP) con-taining $CD5a_{desArg}$[23] was generated by activating complement in normal serum with 5 mg/ml of zymosan A (Sigma Chemical Co., St. Louis, MO) for 60 min at 37°, as previously described.[24] The zymosan was removed by centrifugation (2000 *g* for 15 min).

Isolation and growth of fibroblasts

HDF were isolated aseptically from foreskin obtained from circumcized newborns or from skin removed from children during minor plastic surgery (provided by Dr K. Wilson, Izaak Walton Killam Children's Hospital, Halifax, Canada). The tissue was minced in α^+ minimal essential medium (αMEM; Sigma Chemical Co.) and washed once. The minced tissue was digested with 2 mg/ml collagenase type IV (512 U/mg; Sigma) in αMEM–10% heat-inactivated fetal calf serum (FCS; Hyclone Laboratories, Logan, UT), containing penicillin G–streptomycin (Gibco, Grand Island, NY), by incubation in a shaker (250 r.p.m.) at 37° for 4 hr. After digestion, single cells were collected, washed and cultured in αMEM supplemented with 10% FBS, 2-mercaptoethanol (2-ME) and penicillin G–streptomycin until cells grew to confluence. The cells were passaged until the cultures became homogeneous. Cells at the third to 12th passage were used. They were harvested with 0·05% trypsin/0·02% EDTA (Flow Laboratories, Mississauga, Ontario, Canada) and seeded at 2×10^4 cells in 0·1 ml onto Transwell filters of 5 μm pore size (Costar Corp, Cambridge, MA), which were precoated with 0·01% gelatin overnight. Fibroblasts on the filters were cultured in αMEM with 10% FCS, 2-ME and antibiotics for 5–6 days until they formed a tight monolayer and permeability barrier. The permeability of monolayers was measured prior to migration assay using diffusion of ^{125}I-labelled HSA as described previously.[25] Filters with less than 5% ^{125}I-HSA diffusion in 45 min were used.

Isolation of PMNL

Blood PMNL were isolated as described previously.[25] Briefly,

peripheral venous blood from a healthy donor was collected into heparin (5 U/ml blood) and acid citrate dextrose (1·6 ml/10 ml blood; ACD formula A; Travenol, Malton, Ontario, Canada) anticoagulant. The red blood cells were sedimented by 6% dextran saline (1 part to 5 parts blood) (Travenol). The leucocyte-rich plasma (LRP) was harvested and centrifuged at 150 *g* for 10 min. The pellet containing leucocytes and residual red blood cells was resuspended in Ca^{2+}, Mg^{2+}-free Tyrode's solution with 5% autologous platelet-poor plasma (PPP) and labelled with ^{51}Cr-sodium chromate (40 μCi/ml; Amersham Corp., Oakville, Ontario, Canada) by incubation for 30 min at 37°. The labelled PMNL were separated on discontinuous Percoll (Pharmacia Fine Chemicals, Dorval, Quebec, Canada) gradients (58%/73%), containing 10% PPP, by centrifugation for 30 min at 300 *g*. After two washes with Tyrode's solution containing 0·1% HSA, the PMNL were suspended in RPMI-1640 medium containing 0·5% HSA and 10 mM HEPES (RHSA), pH 7·4, to a final concentration of 2×10^6/ml. This method yielded > 95% pure PMNL with > 98% viability with essentially no red blood cell contamination.

PMNL transfibroblast migration

The migration assay was performed as described previously.[16,25] Briefly, fibroblast monolayers on the filters and the lower compartment were washed with RPMI-1640 and incubated for 4 hr in fresh RPMI-1640 with 10% FCS, or stimulated for 4 hr by addition of cytokine (TNF-α or IL-1α) to the medium. After incubation, the filters were washed on the upper and lower surfaces with RPMI-1640 and transferred to a new, clean well (lower compartment) of a 24-well plate. To this well, 0·6 ml RHSA was added containing chemotactic stimulus (C5a, IL-8 or ZAP). Before immersion of the fibroblast–filter unit in the well, 0·1 ml of medium containing 2×10^5 ^{51}Cr-labelled PMNL was added above the fibroblast–filter unit. After incubation, PMNL migration was stopped by washing the upper surface of the monolayers twice with 150 μl RPMI-1640 to remove non-adherent PMNL. The under surface of the filter was then swabbed with a cotton swab, wetted by ice-cold PBS/0·2% EDTA solution, and the swab combined with the contents of the lower compartment. The cells that dropped off into the lower compartment and were removed by the swab from the under surface of the filter were considered to have migrated. These cells were lysed by addition of 0·5% Triton X-100, and the medium in this compartment, including the swab, were analysed for ^{51}Cr to calculate migration. The PMNL adhesion to the fibroblasts was determined by first washing off the non-adherent PMNL above the monolayer and swabbing the filter under surface, followed by immersion of the fibroblast–filter unit in 0·5 N NaOH to release the ^{51}Cr from the remaining adherent PMNL. The results are expressed as the percentage of the total ^{51}Cr-labelled PMNL added above the fibroblast monolayers that migrated across (i.e. c.p.m. in lower compartment + recovered on swab from filter under surface) or remained adherent to the fibroblast–filter unit (i.e. c.p.m. released by NaOH from the fibroblast–filter unit). All the treatment conditions were performed in triplicate.

Antibody treatment

In some experiments, ^{51}Cr-labelled PMNL were treated for 20 min at room temperature (22°) with saturating concentration of mAb (30–50 μg/ml) before being tested for migration. The antibodies were present throughout the migration assay except when indicated otherwise. In some experiments, ^{51}Cr-labelled PMNL were treated with mAb as above, washed with RHSA containing 10 mM HEPES to remove free antibody, and tested for migration in the absence of antibody. In some cases, fibroblast monolayers were pretreated with mAb for 40 min at 37° and washed three times with RPMI-1640 to remove free mAb, before PMNL were added to the fibroblast monolayers.

Immunofluorescence staining

The leucocyte-rich plasma was prepared and centrifuged as described above. The pellet containing leucocyte and residual red blood cells was washed, resuspended in RHSA (1×10^7 PMNL/ml) with or without C5a (1×10^{-8} M) and incubated at 37° for 30 min. After incubation, the cells were centrifuged and the pellet was resuspended in cold PBS (pH 7·4) containing 0·5% bovine serum albumin and 0·1% azide (PBS/BSA/Az). The cells (0·1 ml, 1×10^6 PMNL) were stained sequentially at 4° (30 min) with mAb (ascites, 1 : 200; pure IgG, 10 μg/ml) in PBS/BSA/Az, and fluorescein isothiocyanate (FITC)-conjugated IgG goat anti-mouse immunoglobulins (IgG, IgA and IgM), (Cappel Labs, Malvern, PA) at 1 : 500 dilution in PBS/BSA containing 10% heat-inactivated goat serum. Between each step, cells were washed three times. After staining, residual red blood cells were lysed with warm 0·84% NH_4Cl and fixed with 1% paraformaldehyde in PBS (Sigma). The fluorescence intensity was measured with a FACScan flow cytometer (Becton Dickinson Immunocytometery Systems, Mountain View, CA) and analysed using PC-LYSYS (Becton Dickinson) software.

Statistical analysis

One-way analysis of variance (ANOVA) or Student's *t*-test were used for analysis of the data. *P*-values < 0.05 were considered to be significant.

RESULTS

Migration of PMNL across fibroblasts in response to C5a

After 5–6 days of culture, fibroblasts became confluent and formed relatively tight monolayers, with diffusion of < 5% of the ^{125}I-HSA added to the upper compartment in 45 min, compared to diffusion across a bare filter of 25–30%. The barrier was sufficient that PMNL did not migrate significantly across the fibroblasts and filter in the absence of a chemotactic factor during a 2-hr period (spontaneous migration = 1–2% of added PMNL). However, PMNL rapidly migrated through the monolayer–filter to the lower compartment when the C5a chemotactic factor was added to the lower compartment, and this was dose-dependent (Fig. 1). The concentration that induced significant PMNL migration during a 90-min period was between 1×10^{-10} M and 1×10^{-8} M, but the maximal response was between 2×10^{-9} M and 1×10^{-8} M. The time-course of PMNL transfibroblast migration to C5a was determined. As shown in Fig. 2a, PMNL migration through the fibroblast–filter unit was detectable at 30 min in the presence of 2×10^{-9} M C5a. The PMNL migrated progressively over a 90-min period, then reaching a plateau. Therefore,

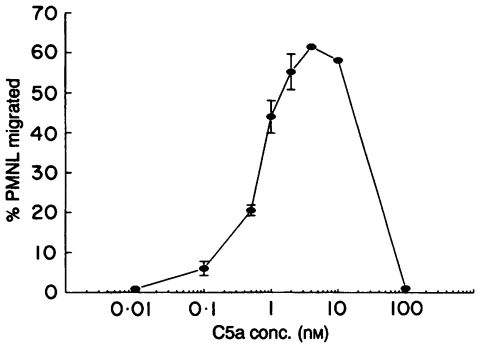

Figure 1. The dose–response of C5a-induced PMNL transfibroblast migration. Human ^{51}Cr-labelled PMNL (2×10^5 in 0·1 ml) was added above fibroblast monolayers, as described in the Materials and Methods. The migration of PMNL across the fibroblast–filter unit was induced by varying concentrations of recombinant C5a in the lower compartment beneath the filter. The migration time was 90 min. Results are expressed as a percentage of added PMNL, which migrated through the fibroblast–filter unit. Values are mean ± SEM of three to nine experiments performed in triplicate.

a 90-min migration time was used in most experiments. To determine if the migration was preceded by PMNL adhesion to the fibroblasts, this was measured at various times. Figure 2b shows that a small but significant increase in PMNL adhesion

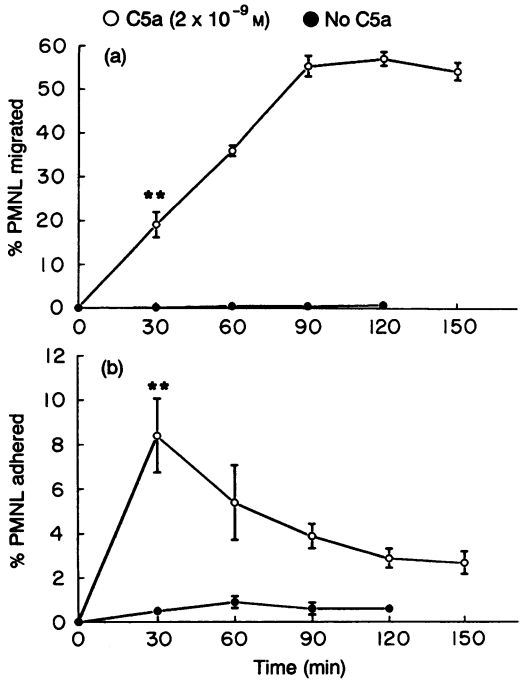

Figure 2. The kinetics of PMNL adhesion to and migration across fibroblast monolayers in response to C5a. The ^{51}Cr-labelled PMNL were added above the fibroblasts and transmigration was measured in the absence of a stimulus or following addition of C5a (2×10^{-9} M) to the lower compartment, as in Fig. 1. The PMNL migration (a) and adhesion (b) through fibroblast monolayers was determined after the incubation times shown. Results are expressed as in Fig. 1. Values are mean ± SD of one representative experiment of two performed in triplicate. $^{**}P < 0.01$ compared to no C5a.

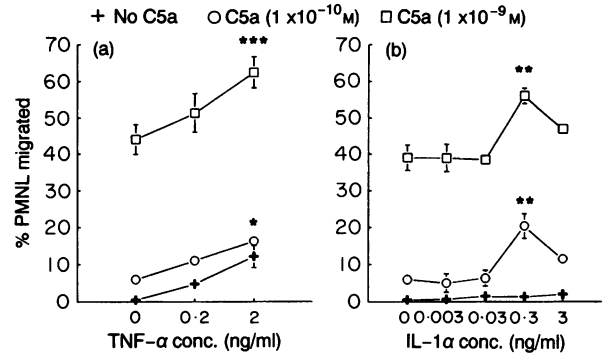

Figure 3. The effect of TNF-α and IL-1α treatment of fibroblasts on PMNL transmigration to C5a. The fibroblast monolayers were stimulated with various concentrations of TNF-α or IL-1α for 4 hr, then washed and ^{51}Cr-labelled PMNL added above the monolayers. Migration was induced by the concentration of C5a indicated, added to the compartment beneath the fibroblast–filter unit. Migration was for 90 min. Results are expressed as in Fig. 1. Values are mean ± SEM of three to five experiments in each group. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$, compared to fibroblasts not treated with TNF-α or IL-1α. The error bars for some values are smaller than the size of the symbols.

occurred at 30 min, which gradually decreased with time as the PMNL migrated.

Effect of TNF-α and IL-1α treatment of fibroblasts on migration of PMNL in response to C5a

Previously we reported that treatment of fibroblasts with TNF-α, but not IL-1α, caused PMNL migration across fibroblast monolayers in the absence of any added chemotactic factor.[16] In this study, we investigated whether treatment of the fibroblasts with TNF-α and IL-1α may modulate PMNL transfibroblast migration in response to C5a. The fibroblast monolayers were stimulated with TNF-α or IL-1α for 4 hr before adding ^{51}Cr-labelled PMNL above the monolayer–filter unit. As shown in Fig. 3, prestimulation of fibroblasts with TNF-α or IL-1α significantly enhanced PMNL migration across the monolayer–filter unit in response to C5a. This enhancement of migration was dose-dependent with TNF-α and was significant at the higher but suboptimal C5a concentration tested, i.e. 1×10^{-9} M. This effect was largely additive because TNF-α treatment of the fibroblasts alone induced PMNL transmigration, as reported previously.[16] In contrast, IL-1α treatment of the fibroblast monolayers alone did not induce PMNL transfibroblast migration, but at a concentration of 0·3 ng/ml IL-1α significantly enhanced C5a-induced transfibroblast migration at both C5a concentrations tested. It is not clear why there was such a narrow optimal concentration of IL-1α, i.e. why higher dose treatment (3 ng/ml) of fibroblasts with IL-1α decreased or reversed the enhancement of PMNL migration (Fig. 3).

The β_2 (CD11–CD18) and β_1 (CD29) integrins mediate PMNL transfibroblast migration

The β_2-integrin CD11–CD18 complex is required for virtually

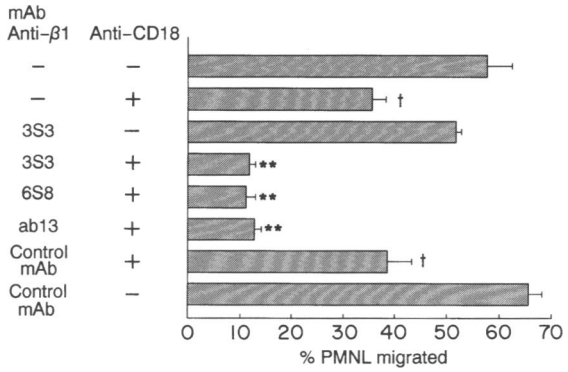

Figure 4. The effect of mAb to CD18 and β_1-integrin (CD29) on C5a-induced transfibroblast migration of PMNL. The ^{51}Cr-labelled PMNL were treated (20 min, 22°) with mAb to CD18 (60.3 or R15.7, 30 μg/ml) or a combination of mAb 60.3 plus mAb to β_1-integrin (mAb 3S3, 40 μg/ml, or 6S8, 1 : 100 ascites, or ab13, 50 μg/ml) or control mAb (anti-CR1, 543, or anti-CD14, 3C10). C5a (2×10^{-9} M) was used to induce migration as in Fig. 1, for 90 min. In the absence of C5a, PMNL migration was 2·1 ± 0·3%, with all treatment conditions. Values are mean ± SEM of three experiments in each group, performed in triplicate. $^{**}P < 0.01$ compared to anti-CD18-treated group; $\dagger P < 0.05$ compared to control mAb-treated group.

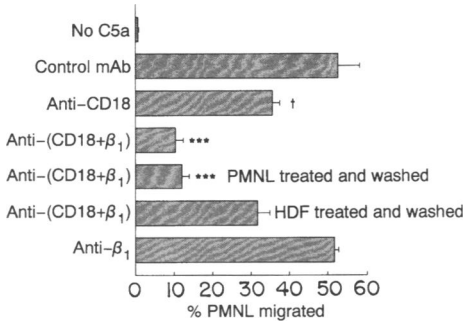

Figure 5. The β_1-integrins on PMNL but not on dermal fibroblasts are required for CD18-independent transfibroblast migration. The ^{51}Cr-labelled PMNL were pretreated (20 min, 22°) with mAb 60.3 (anti-CD18) or mAb 3S3 (anti-β_1-integrin), alone or in combination. Control mAb to CD14 (3C10) or to CR1 (543) was used. Where indicated, PMNL were treated with a combination of the mAb to CD18 and β_1-integrin, then washed twice and added above the fibroblasts together with mAb to CD18. Alternatively, the fibroblast monolayers were treated with mAb to β_1-integrin for 40 min at 37° and washed before PMNL, treated with anti-CD18 mAb, were added above the fibroblast monolayers. The migration was induced by C5a (2×10^{-9} M) for 90 min. Values are mean ± SEM of three to six experiments in each group. $^{***}P < 0.001$ compared to PMNL treated with mAb to CD18 alone; $\dagger P < 0.05$ compared to PMNL treated with control mAb.

all (85–90%) PMNL migration in response to C5a across human unbilical vein endothelium (HUVE)[15,26] or across TNF-α-stimulated fibroblasts, as reported previously.[16] Here we examined the role of this integrin complex in the PMNL response to C5a. The ^{51}Cr-labelled PMNL were treated, before being added above the monolayers, with mAb 60.3 to CD18, which blocks the adhesion–migration function of the CD11–CD18 complex.[17] As shown in Fig. 4, treatment of PMNL with mAb 60.3 only partially inhibited PMNL migration across the fibroblast monolayers in response to C5a, i.e. about 38% of the migration response. In order to confirm whether the effect of mAb 60.3 to CD18 integrin in this experimental system was specific, another mAb, i.e. R15.7, which also functionally inhibits CD18-mediated PMNL migration through vascular endothelium,[27] was also tested. Treatment of PMNL with mAb R15.7 also only partially (35–40%) inhibited PMNL transfibroblast migration, i.e. similar to the effect of mAb 60.3. When control mAb reactive with CR1 (mAb 543) or CD14 (mAb 3C10) on PMNL were used to treat PMNL, there was no effect on PMNL migration. The results indicate that the mechanism of C5a-induced PMNL migration across fibroblast monolayers is only partially CD18-dependent and must also involve one or more CD18-independent mechanisms.

In addition to the CD11–CD18 adhesion molecules, PMNL also express β_1-integrins, although at a much lower level.[6,9,28] Major ligands for the β_1-integrins are fibronectin, laminin and collagen,[11,12] some of which can be produced by fibroblasts.[5] Therefore, we investigated the role of β_1-integrins in PMNL transfibroblast migration in response to C5a. The PMNL were treated for 20 min with both mAb 60.3, CD18, to block the CD11–CD18 mechanism, and mAb 3S3 to 6S8 or ab13, each of which recognize the β_1-integrin (CD29) chain and block the adhesion function of these integrins[18] (see the Materials and Methods). Figure 4 shows that each of these mAb inhibited the migration of PMNL in response to C5a across the fibroblasts if the CD11–CD18 complex was also blocked by mAb 60.3. This inhibition was 65–68%. Interestingly, treatment of PMNL with mAb to β_1-integrins alone had no significant effect on PMNL transfibroblast migration in response to C5a, suggesting that the CD11–CD18 mechanism is dominant, although β_1-integrin could function as an alternative pathway for PMNL transfibroblast migration. It is important to point out that treatment with control mAb reactive with CR1 (mAb 543) or with CD14 (mAb 3C10) on the PMNL did not inhibit the migration of anti-CD18 treated PMNL (pooled results; Fig. 4).

The β_1-integrin on PMNL, but not on fibroblasts, is required for CD18-independent migration

We investigated whether the effect of anti-β_1-integrin mAb was on the PMNL or on the fibroblast, because β_1-integrins are present on both cell types.[5,6,10–12,28] The PMNL were treated with mAb to CD18 (60.3) in combination with mAb to β_1-integrins (3S3) and then washed to remove free antibody. Monoclonal antibody to CD18 alone was added back to ensure that CD11–CD18 would be blocked during the assay, and these PMNL were added onto the fibroblast monolayers. Alternatively, the fibroblast monolayers were pretreated with mAb to β_1-integrins (3S3) and washed before the anti-CD18 mAb-treated PMNL were added. As shown in Fig. 5, the migration of PMNL treated with mAb to β_1-integrin, in the presence of mAb to CD18, was still inhibited. In contrast, treatment of the fibroblast monolayers with mAb to β_1-integrin had no effect on CD18-independent PMNL transfibroblast migration (Fig. 5). The results indicate that β_1-integrins on the PMNL but not on

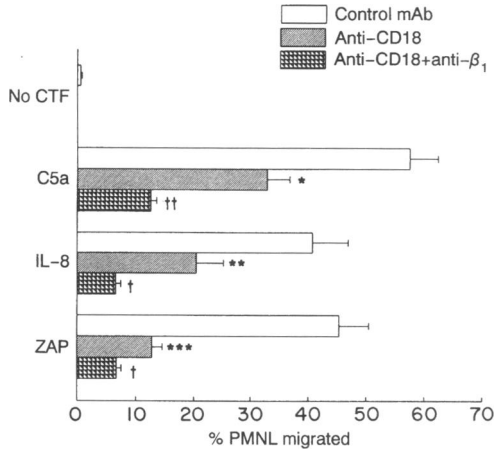

Figure 6. The effect of chemotactic factors on PMNL transfibroblast migration. The ^{51}Cr-labelled PMNL were pretreated (20 min, 22°) with mAb to CD18 (60.3), or a combination of mAb to CD18 and β_1-integrin (3S3). Monoclonal antibody to CD14 (3C10) or to CR1 (543) was used as control. Chemotactic factors (CTF) C5a (2×10^{-9} M), IL-8 (6×10^{-9} M/ml) or 2% ZAP ($C5a_{desArg}$) were added into the lower compartment beneath the fibroblast–filter unit. The migration was 90 min as in Fig. 1. Values are mean ± SEM of three to four experiments, performed in triplicate in each group. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$, compared to PMNL treated with control mAb; $\dagger P < 0.05$; $\dagger\dagger P < 0.01$, compared to anti-CD18 treatment alone.

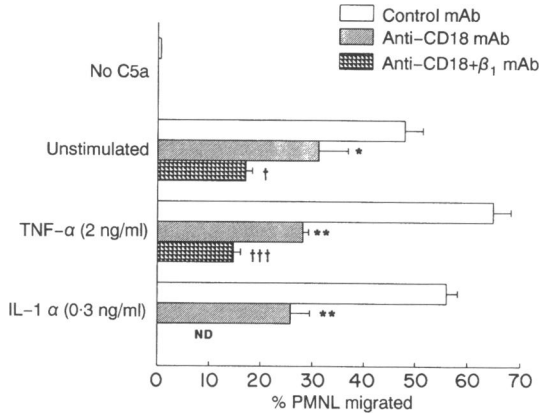

Figure 7. The effect of TNF-α and IL-1α on CD18 or β_1-integrin-mediated transfibroblast migration of PMNL in response to C5a. The ^{51}Cr-labelled PMNL were pretreated with mAb to CD18 (60.3) for 20 min, or a combination of mAb 60.3 with mAb to β_1-integrins (3S3), at room temperature (22°). Control mAb to CD14 (3C10) or to CR1 (543) was used. Where indicated, the fibroblast monolayers were treated (4 hr) with TNF-α (2 ng/ml) or IL-1α (0·3 ng/ml), as in Fig. 3. The migration was induced by addition of C5a (1×10^{-9} M) into the lower compartment. The migration time was 90 min. Results are expressed as in Fig. 1. Values are mean ± SEM of three to six experiments in each group. $*P < 0.05$; $**P < 0.01$, compared to control antibody-treated group; $\dagger P < 0.05$; $\dagger\dagger\dagger P < 0.001$, compared to anti-CD18 antibody-treated group. ND, not determined.

the fibroblasts mediate CD18-independent PMNL transfibroblast migration.

Comparison of PMNL transfibroblast migration induced by various chemotactic factors

We further investigated whether the CD18-independent transfibroblast migration of PMNL was unique to C5a or was a general property of PMNL activated by chemotactic factors. Migration of PMNL through the fibroblast monolayers was also observed when IL-8 or ZAP, a source of the more stable $C5a_{desArg}$ chemoattractant,[23,24] was used as stimulus (Fig. 6). The optimal concentration for PMNL transfibroblast migration with IL-8 was 50 ng/ml (6×10^{-9} M), and with ZAP was 2% (data not shown). As shown in Fig. 6, recombinant C5a at 2×10^{-9} M induced a greater maximal transfibroblast migration of PMNL than ZAP or IL-8. The relative proportion of CD18-dependent and CD18-independent transfibroblast migration of PMNL differed somewhat with these stimuli. The ZAP-induced PMNL transfibroblast migration was more CD18 dependent, i.e. 72% inhibition by anti-CD18 treatment, than in response to IL-8 (51% inhibition) or to C5a (43% inhibition). The CD18-independent PMNL migration component in response to each of these chemotactic stimuli was inhibited further by treating the PMNL with mAb 3S3 to β_1-integrin as well, suggesting that β_1-integrins also plays a role in PMN migration induced by these factors. Interestingly, IL-8- and ZAP-induced PMNL transfibroblast migration was almost completely inhibited by mAb to both CD18 and β_1-integrin (5–7% migration remained), which was distinct from the response to C5a, where a significant migration component (12·4 ± 1·0% of PMNL migrated) was not inhibited (Figs 4 and 6).

The effect of cytokines on CD11–CD18 and β_1-integrin-mediated transfibroblast migration of PMNL

As described above, pretreatment of fibroblast monolayers with TNF-α or IL-1α for 4 hr increased PMNL migration across the monolayers in response to C5a (Fig. 3). We further investigated the effect of these cytokines on CD18 and β_1-integrin-mediated transfibroblast migration of PMNL in response to C5a. For these experiments, a suboptimal concentration of C5a (1×10^{-9} M) was used so that enhancement of PMNL migration by IL-1 or TNF-α was observed, as in Fig. 3. As shown in Fig. 7, the cytokine-enhanced transfibroblast migration of PMNL was inhibited by 57% by mAb 60.3 to CD18 with TNF-α treatment, and by 54% with IL-1α treatment of the fibroblast monolayers. This was more CD18-dependent migration than observed with unstimulated fibroblasts, on which mAb to CD18 inhibited only 35%. However, treatment of fibroblasts with TNF-α did not affect the proportion of β_1-integrin-mediated PMNL migration, because addition of mAb to β_1-integrin (3S3) to the mAb to CD18 inhibited migration further by comparative proportions (48% versus 45% was the β_1-integrin component of the CD18-independent migration response; Fig. 7). The results suggest that TNF-α treatment of fibroblasts enhanced the CD18-dependent, but not the β_1-integrin-mediated, migration of PMNL through fibroblast monolayers in response to C5a.

Integrins VLA-5 and VLA-6 are involved in transfibroblast migration of PMNL

We further investigated the specific β_1-integrins on the PMNL

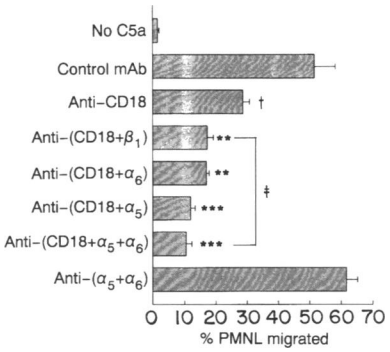

Figure 8. The role of VLA-5 and VLA-6 in β_1-integrin-mediated transfibroblast migration of PMNL. The ^{51}Cr-labelled PMNL were pretreated (20 min, 22°) with mAb to CD18 (60.3) alone or mAb to β_1-integrin (3S3), α_5 of VLA-5 (ab16; 50 μg/ml) or to α_6 of VLA-6 (450-30A1; 1 : 100) in combination as indicated. Control mAb to CD14 (3C10) or to CR1 (543) was used. All mAb were present during the migration (90 min) induced by C5a (2×10^{-9} M). Values are mean ± SEM of four to five experiments, performed in triplicate. $^{**}P < 0{\cdot}01$; $^{***}P < 0{\cdot}001$, compared to PMNL treated with anti-CD18 alone; $\dagger P < 0{\cdot}05$ compared with control mAb group. $\ddagger P < 0{\cdot}05$.

involved in CD18-independent transfibroblast migration induced by C5a. We employed adhesion-function blocking mAb to the α_5-chain of VLA-5 (ab16) and to α_6 of VLA-6 (mAb 450-30A1 or GoH3). These are the only known β_1-integrins on PMNL.[6,9,28] The PMNL were treated with the anti-α_5 and -α_6 mAb alone or in combination with mAb to CD18. Figure 8 shows that both anti-α_5 (ab16) and anti-α_6 (450-30A1) mAb significantly inhibited C5a-induced PMNL transfibroblast migration when CD18 on the PMNL was also blocked. In fact, mAb to α_5 and α_5 plus α_6 were slightly more inhibitory than the mAb to the β_1-integrin chain (3S3) used in these paired experiments. We also used mAb GoH3, which is a rat mAb to the α_6-chain of VLA-6, and observed comparable inhibition to that seen with mAb 450-30A1 (data not shown). In contrast, treatment of PMNL with mAb to α_5 (ab16) or α_6 (mAb 450-30A1), alone or in combination, did not inhibit PMNL migration through fibroblast monolayers, if the CD11–CD18 complex was not blocked (Fig. 8). In these experiments, treatment of PMNL with control mAb (3C10 anti-CD14 or mAb 543 anti-CR1) alone or in combination with mAb or CD18, did not inhibit the C5a-induced CD18-independent PMNL migration (data not shown).

Expression of VLA-5 and VLA-6 on PMNL

The presence of VLA-5 and VLA-6 receptors on human PMNL have been described in a few reports,[6,9,10,28] but some discrepancy exists in the literature.[12,29] The functional assays in this study indicated that VLA-5 and VLA-6 were involved in human PMNL transfibroblast migration. We performed immunofluorescence analysis using the mAb employed above and observed that VLA-5 and VLA-6 were expressed on the preparations of PMNL employed, although at relatively low level. The mean channel fluorescence intensity (MFI) on staining with irrelevant control mAb (3H11B9; anti-pertussis toxin) was 9·72 ± 0·42 (n = 2), while with mAb to α_5 (ab16) this increased to 14·59 ± 0·04 (n = 2), and with mAb to α_6 (450-30A1) the MFI was 31·38 ± 0·02 (n = 2). On staining with mAb to β_1-integrin (3S3) the MFI was 57·63 ± 1·4 (n = 4). For comparison, MFI of staining with mAb to CD18 (mAb 60.3) was 290. Activation of PMNL with C5a did not change the staining intensity for α_5, α_6 and β_1-integrins, although, as expected, the staining intensity with anti-CD18 (mAb 60.3) increased by threefold (MFI = 855).

DISCUSSION

The mechanisms of PMNL interaction with and migration through connective tissue are relatively poorly understood. Here we have shown that PMNL do not adhere appreciably to dermal fibroblasts and do not migrate through fibroblast monolayers unless the PMNL is activated by a chemotactic factor, such as C5a, C5a$_{desArg}$ or IL-8 (Figs 1, 2 and 6). During PMNL migration, PMNL adhesion to the fibroblasts increased transiently. This may be due to activation of adhesion molecules on PMNL for ligand binding by chemotactic factors.[2,3,11,30] The transfibroblast migration of PMNL in response to C5a is very dose-dependent (Fig. 1), with an optimal response observed in a narrow range. This is similar to the chemotactic response to most chemotactic factors in a Boyden filter type system in the absence of a cell barrier.[23,31,32]

The PMNL migration response to C5a was also influenced by the fibroblasts, because activation of fibroblasts for brief periods (4 hr) by the proinflammatory cytokines IL-1 and TNF-α enhanced the migration induced by suboptimal concentrations of C5a (Fig. 3). Such treatment up-regulates ICAM-1 expression on fibroblasts.[16,33] ICAM-1 is a major ligand for LFA-1 and Mac-1 of the CD11–CD18 complex on PMNL, and is are important for PMNL migration.[1,2,14,15] However, this action of IL-1 and TNF-α alone does not account for all of the observed migration, because the degree of ICAM-1 up-regulation on the fibroblasts by the IL-1 and TNF-α concentrations used was comparable, as reported previously,[16] yet TNF-α treatment alone induced an increase in PMNL transendothelial migration but IL-1 did not (Fig. 3). Furthermore, the dose–response curves for IL-1 and TNF-α were quite different. Thus other mechanisms, such as chemokine production by the fibroblasts, may also play a role in the differential responses to TNF-α and IL-1.[34,35]

It is known that PMNL migration across vascular endothelial cells is mediated by the CD11–CD18 (β_2-integrins) on PMNL, which recognize the ligands ICAM-1 and ICAM-2 on endothelial cells.[1,2,14] However, the mechanisms of PMNL migration in the extravascular connective tissue are not clear, although it has been shown that PMNL adhesion to ECM proteins involves both CD11–CD18 integrins and β_1-integrins.[6–10] In this study, we found that in response to C5a, C5a$_{desArg}$ (ZAP) or IL-8, PMNL migration through fibroblast monolayers was only partially mediated by CD18 integrins, because treatment of PMNL with mAb to CD18 inhibited PMNL transfibroblast migration by only 35–70%, depending on the stimulus (Figs 3 and 6). This is in marked contrast to migration through endothelium in response to these chemotactic factors, where PMNL migration is inhibited by 85–95% by mAb to CD18,[14,15,26] or through a dermal fibroblast barrier activated by TNF-α, as previously reported using the current

assay system.[16] Thus the PMNL migration observed here must involve CD18-independent mechanisms not required during migration through endothelium. Furthermore, the chemotactic factors tested here must activate additional migration mechanisms on PMNL, which may interact with fibroblasts or ECM. The results shown here indicate that the β_1-integrins (CD29) on the PMNL serve as one alternative migration mechanism, because three different mAb to the β_1-integrin chain inhibited this migration, e.g. by 65% relative to anti-CD18-treated PMNL (about 40% of the total PMNL migration response) (Fig. 4). This role of the β_1-integrins was detectable only when the CD18 function was blocked by mAb, since mAb to β_1-integrins alone did not inhibit migration (Figs 4 and 5). This suggests that normally the CD11–CD18 pathway is probably dominant in mediating PMNL migration through fibroblast barriers. The results in Fig. 7 shows that TNF-α treatment of the fibroblasts could increase both the PMNL transfibroblast migration to C5a and the proportion of migration dependent on CD11–CD18. This may be secondary to increased ICAM-1 expression on the fibroblasts.[16,36] However, TNF-α treatment did not alter the contribution of β_1-integrins to the PMNL migration response.

From the data in Fig. 4, it is clear that a minor portion (15–20% of the migration response or 10–12% of the PMNL migrated) was not inhibited by a combination of mAb to CD18 and β_1-integrin. Although it is possible that this was due to incomplete mAb blocking, saturating concentrations of mAb were used and increasing mAb concentration did not inhibit migration further (data not shown). Thus other mechanisms may also contribute to PMNL migration across fibroblast barriers, which yet need to be defined. In preliminary experiments, blocking mAb to the known molecules on PMNL involved in PMNL interaction with endothelium, such as L-selectin, the sialyl lewis^x carbohydrates or the leucocyte response integrin,[1,2,37] did not inhibit this CD18 and β_1-integrin component of PMNL migration (our unpublished observations). Furthermore, mAb to E-selectin, which inhibits PMNL adhesion to cytokine-activated endothelium and partially inhibits transendothelial migration,[1,26] had no effect on chemotactic factor-induced PMNL migration through fibroblasts (our unpublished observations). In this context, the recent report by Senior *et al.*,[37] demonstrating that the 'leucocyte response integrin' on PMNL can mediate chemotaxis to entactin, may be relevant and deserves further investigation. Thus, overall, PMNL migration through fibroblasts utilizes distinct mechanisms from migration through endothelial cells, i.e. not only CD11–CD18 but also β_1-integrins and possibly additional pathways. These differences may be related to the ECM proteins produced by fibroblasts, rather than cell surface-bound adhesion molecules such as ICAM-1, ICAM-2 and E-selectin, which on endothelium are involved in PMNL adhesion and migration.[1,2]

The β_1-integrin family is composed of numerous members referred to as very late antigens (VLA integrins).[11,12] Distinct α-chains associate with the common β_1-chain to provide a variety of ligand specificities for VLA integrins. Of these, VLA-5 ($\alpha_5\beta_1$) and VLA-6 ($\alpha_6\beta_1$) are expressed on human PMNL and appear to mediate the adhesion of PMNL to ECM proteins such as fibronectin and laminin, the major known ligands for VLA-5 and VLA-6, respectively.[6,9,12,28] In this study, we confirmed by immunofluorescence flow cytometry that both VLA-5 and VLA-6 are present on human PMNL, although at relatively low levels, much lower than CD11–CD18 (see the Results).[6,9,28] Our results indicate that both VLA-5 and VLA-6 are involved in CD18-independent fibroblast migration of PMNL, since specific mAb for α_5 and α_6 inhibited this migration. The findings that mAb to either α_5 or α_6 chains was as effective for inhibition of migration as blocking the common β_1-chain (Fig. 8) suggest that both VLA-5 and VLA-6 are involved in CD18-independent PMNL migration, and that both are required for adequate ligand binding and/or signalling of the PMNL for migration. These integrins may be working sequentially to account for the observed effects of the α-chain specific mAb. Alternatively, since the level of expression of both VLA-5 and VLA-6 is low on PMNL, both VLA-5 and VLA-6 may be required to achieve sufficient ligand binding and/or signalling to support migration. The finding that, upon treatment of PMNL with both mAb to α_5 and α_6 in the presence of mAb to CD18, inhibition was greater than with mAb to the β_1-chain suggests that the α subunits may play a more important role in migration than the β subunit, or that the mAb to the α-chain recognize epitopes closer to the functional domain. It is difficult to exclude the alternative possibility that ligation of either VLA-5 or VLA-6 on the PMNL by mAb may generate an inhibitory signal in PMNL for migration. However, this is unlikely since migration of PMNL was not inhibited by treatment with mAb to VLA-5 plus VLA-6 (Fig. 8).

The migration of PMNL *in vivo* to inflammation can be induced by a variety of inflammatory mediators. The chemotactic factors C5a, $C5a_{desArg}$ and IL-8 are important PMNL recruiting factors in various inflammatory diseases.[32,35,38] All three of these chemotactic factors induced PMNL transfibroblast migration, but C5a and IL-8 induced migration to a relatively greater degree via the β_1-integrin-dependent mechanism (about 35% of the response) than did $C5a_{desArg}$ in ZAP (Fig. 6). This may be related to the fact that IL-8 and C5a have similar potency in activating PMNL and inducing chemotaxis,[32,39] while $C5a_{desArg}$, on a molar basis, is a weaker PMNL agonist and chemotactic factor.[23,32]

In summary, to our knowledge, this study is the first to show that PMNL migrate across a fibroblast barrier in response to chemotactic factors and that the β_1-integrins (CD29) VLA-5 and VLA-6 can mediate PMNL migration. This is in addition to migration via the CD11–CD18 integrin pathway and possibly another mechanism yet to be identified, especially in response to C5a. The results demonstrate that PMNL migration through connective tissue barriers and matrix involves distinct mechanisms from migration across vascular endothelium. These findings suggest further investigation is warranted along these lines, to understand better the migration of leucocytes in inflamed tissues and their retention at sites of inflammation in disease states, in which PMNL play a prominent role.

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