PECAM-1, α6 integrins and neutrophil elastase co-operate in mediating neutrophil transmigration

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Summary

The heterogeneous nature of the perivascular basement membrane (composed primarily of laminin and collagen type IV) suggests the existence of an elaborate array of adhesive interactions and possibly proteolytic events in leukocyte migration through this barrier. In this context, blockade of α6 integrins (laminin receptors), neutrophil elastase (NE) or both inhibited neutrophil migration through interleukin-1β (IL-1β)-stimulated mouse cremasteric venules, as observed by intravital microscopy. Furthermore, analysis of tissues by confocal microscopy indicated a synergistic role for α6 integrins and NE in mediating neutrophil migration through the perivascular basement membrane. Using a combined in vitro and in vivo experimental approach, the findings of this study also suggest that α6 integrins and NE are mobilized from intracellular stores to the cell surface of transmigrating mouse neutrophils, although these events occur via mechanisms dependent on and independent of platelet/endothelial-cell adhesion molecule 1 (PECAM-1, CD31), respectively. Despite different regulatory mechanisms, blockade of α6 integrins or NE inhibited migration of murine neutrophils through laminin-coated filters in vitro. Collectively, the findings suggest that, whereas regulation of the expression of α6 integrins and NE occur via different adhesive mechanisms, these molecules might act in a co-operative manner in mediating neutrophil migration through venular walls, in particular the perivascular basement membrane.

Key words: CD31, Transmigration, Protease, Mice, Neutrophils, Integrins

Introduction

Leukocyte migration from the vascular lumen to the extravascular tissue is a fundamental requirement in the host's inflammatory response to bacterial invasion and tissue injury. It is currently accepted that this is a multistep process in which leukocytes undergo rolling and tight adhesion to venular endothelium within the vascular lumen, followed by traversing the endothelial cell barrier and its associated perivascular basement membrane (PBM) (Vestweber and Blanks, 1999; Dunon et al., 1996; Yadav et al., 2003). Although we know much about the intricate details of the molecular and cellular interactions that mediate the early interactions of leukocytes with endothelial cells (Alon and Feigelson, 2002), we know less about the mechanisms that mediate migration of leukocytes through venular walls, in particular migration through the PBM (Yadav et al., 2003).

PBMs are sheet-like structures composed of several extracellular matrices that provide structural support for vascular endothelium. The lateral organization of the PBM proteins, primarily two independent networks of laminin and collagen-type-IV molecules (Timpl, 1996), together with its heterogeneous nature suggests that an elaborate array of adhesive interactions and possibly proteolytic events might be involved in leukocyte passage across this barrier. Indeed, both adhesion molecules and proteases have been implicated in this response, although the role of the latter remains contentious. With respect to adhesion molecules, one that has been associated with leukocyte migration through the PBM is platelet/endothelial-cell adhesion molecule 1 (PECAM-1/CD31), a member of the immunoglobulin superfamily that is expressed on the surface of platelets, leukocytes and endothelial cells, where its expression is largely concentrated at the intercellular junctions (Newman, 1997). This molecule has now been linked to many biological functions (Jackson, 2003) but PECAM-1 was the first endothelial cell junctional molecule shown to be specifically involved in leukocyte transendothelial cell migration in vitro and in vivo (Muller et al., 1993; Bogen et al., 1994; Vaporciyan et al., 1993), and, more recently, leukocyte migration through the PBM (Liao et al., 1995; Wakelin et al., 1996; Duncan et al., 1999; Thompson et al., 2001). In addition to PECAM-1, we have found evidence for the involvement of the integrin α6β1, the principal leukocyte receptor for laminin, in leukocyte migration through the PBM in vivo (Dangerfield et al., 2002). Furthermore, by demonstrating that a PECAM-1/PECAM-1 homophilic interaction mediates upregulation of integrin α6β1, we have provided a mechanism by which PECAM-1 might mediate leukocyte migration through the PBM (Dangerfield et al., 2002).

Leukocyte proteases such as the serine protease neutrophil elastase (NE) have also been implicated in leukocyte migration through the PBM, although there are conflicting reports on this

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issue (Delclaux et al., 1996; Huber and Weiss., 1989). Indeed, studies using NE-deficient mice have failed to demonstrate a direct role for NE in neutrophil migration in vivo (Belaaouaj et al., 1998; Tkalcevic et al., 2000; Young et al., 2004). However, because the latter failure might be due to developmental redundancy and there is evidence for increased expression of NE at the leading edge of transmigrating leukocytes (Cepinskas et al., 1999), we hypothesised that NE might act in a functionally co-operative manner with integrin α6β1 to mediate leukocyte migration through the PBM. Hence, to extend our previous work, we have directly investigated the effect of α6-integrin blockade and NE inhibition in leukocyte transmigration in vivo, and have compared the role of PECAM-1 in regulation of expression of these molecules. Our results indicate that, although integrin α6β1 and NE are upregulated on the cell surface of transmigrating neutrophils via different adhesive mechanisms (PECAM-1-dependent and -independent mechanisms, respectively), both molecules appear to facilitate neutrophil migration through the PBM component of venular walls, apparently acting in a synergistic manner. Furthermore, by investigating the role of NE using the approaches of pharmacological blockade and genetic deletion in parallel, this study provides clear indications of developmental redundancy in NE-deficient mice.

Materials and Methods

Animals

Wild-type (WT) C57BL/6 mice (~25 g weight) were purchased from Harlan-Olac (Bicester, UK). PECAM-1-deficient [knockout (KO)] mice and NE KO mice were gifts from T. W. Mak (Amgen Institute, Toronto, Canada) and S. Shapiro (Harvard Medical School, Boston, MA, USA), respectively.

Intravital microscopy

Intravital microscopy on the mouse cremaster muscle was performed as previously described (Thompson et al., 2001). Briefly, male mice were injected intravenously (i.v.) with saline, monoclonal antibody (mAb) GoH3 against α6 integrins (IgG2a; BD Biosciences, Cowley, UK) (Sonnenberg et al., 1987) or an isotype-matched control mAb (IgG2a; Pharmingen, Oxford, UK) (all at 3 mg kg^{-1}), 15 minutes before intrascrotal (i.s.) administration of recombinant murine IL-1β (30 ng per mouse; R&D, Abingdon, UK) or saline (400 μl per mouse). 2 hours later, mice were anaesthetized by intraperitoneal (i.p.) ketamine (Ketalar; 100 mg kg^{-1}; Parke-Davis, Eastleigh, UK) and xylazine (Rompun; 10 mg kg^{-1}; Bayer, Bury St Edmunds, UK), placed on a custom-built, heated (37°C) microscope stage and the jugular vein was cannulated for administration of either saline or the specific NE inhibitor ONO-5046 [ONO Pharmaceuticals, Japan (Kawabata et al., 1991); 50 mg kg^{-1} bolus followed by continuous infusion of 50 mg kg^{-1} $hour^{-1}$ using a syringe pump (Harvard Instruments)]. The cremaster muscle was then surgically exteriorized and events within the microvascular bed were observed using an upright fixed-stage microscope (Zeiss Axioskop FS, Carl Zeiss, Welwyn Garden City, UK) for 2 hours. Leukocyte responses of rolling, firm adhesion and extravasation in postcapillary venules of 20-40 μm diameter were quantified as previously described (Thompson et al., 2001). Leukocyte transmigration was defined as the number of leukocytes in the extravascular tissue across a 200 μm vessel segment and within 50 μm of the vessel of interest. Of relevance, we have previously found that >90% of transmigrated leukocytes in IL-1β-stimulated cremaster muscles (4 hour reaction), as analysed by electron microscopy, are neutrophils (Thompson et al., 2001).

Immunostaining of tissues

Cremaster muscles were exteriorized, as detailed above, dissected away from the animal and fixed in 4% paraformaldehyde (PF). Tissues were blocked and permeabilized in PBS supplemented with 20% horse serum and 0.5% Triton X-100. To investigate the expression of α6 integrins, tissues were incubated with the primary mAb GoH3 at 4°C for 24-48 hours, followed by incubation with a goat anti-rat Alexa-Fluor-488-conjugated secondary antibody (Ab; Molecular Probes, Eugene, OR) for 3 hours at room temperature (RT). For studies in which the objective was to localize the position of transmigrating leukocytes within the vessel wall, tissues were stained for PECAM-1 [as a marker of endothelial cells, using an allophycocyanin (APC)-conjugated rat anti-mouse anti-PECAM-1 Ab (BD Biosciences, Oxford, UK)], laminin 10 [as a marker for the PBM, using a rabbit polyclonal Ab against mouse laminin-α5-chain (a gift from L. Sorokin [University of Lund, Sweden]), detected using a goat anti-rabbit Alexa-Fluor-555-conjugated secondary Ab (Molecular Probes, Eugene, OR)] and CD11b [as a marker for leukocytes, using an Alexa-Fluor-488-conjugated rat mAb against mouse CD11b (BD Biosciences, Oxford, UK)]. Appropriate control Abs were used in parallel with the specific primary Abs. Samples were viewed using a Zeiss LSM 5 Pascal confocal laser-scanning microscope equipped with argon and helium-neon lasers (Zeiss, Welwyn Garden City, UK).

Purification of mouse neutrophils

Mouse blood neutrophils were purified as previously described (Thompson et al., 2001). Briefly, blood was collected from donor mice by cardiac puncture into acid citrate dextrose. After sedimentation of erythrocytes with 6% dextran (Amersham Pharmacia Biotech, Little Chalfont, UK), neutrophils were purified using a two-layer Percoll gradient (80% over 64%), yielding neutrophil preparations of >90% purity. Bone-marrow neutrophils were isolated from wash-outs of femurs of mice using two sequential three-layer Percoll gradients (75%, 65% and 52%), yielding neutrophil preparations of >80% purity.

Stimulation and immunofluorescence imaging of mouse neutrophils

96-well plates were coated overnight at 4°C with different combinations and concentrations of PECAM-1, intercellular adhesion molecule 1 (ICAM-1) and interleukin 8 (IL-8). Briefly, using a 10 μg ml^{-1} solution of each adhesion molecule, wells were coated with either 100% recombinant mouse PECAM-1 (gift from B. Imhof, Centre Medical Universitaire, Geneva, Switzerland) or recombinant human ICAM-1 (R&D, Abingdon, UK). Alternatively, wells were coated with 80% ICAM-1 and 20% PECAM-1 (maintaining the total concentration of the two molecules in the coating solution at 10 μg ml^{-1}), with some wells also being coated with human IL-8 (using solutions of 1 μg ml^{-1} or 10 μg ml^{-1}; PromoCell, Heidelberg, Germany). Control wells were coated with bovine serum albumin (BSA) with or without IL-8. Neutrophils were added to protein-coated wells (2×10^5 per well) and incubated at RT for 30 minutes. In some experiments, the rat anti-human ICAM-1 mAb 6.5B5 (10 μg ml^{-1}; purified from supernatants of hybridoma cell line in house), the rat mAb GAME 46, against mouse β2 integrins (10 μg ml^{-1}; Pharmingen, Oxford, UK), or appropriate isotype-control mAbs were also added to the wells. At the end of the reaction time, attached cells were gently removed after the addition of EDTA (final concentration 0.02%) and samples in each well were fixed by the addition of 2% PF and immediately immunostained for analysis by flow cytometry or confocal microscopy as detailed below.

For flow cytometry, cell suspensions were incubated with the mAb GoH3 against α6 integrins or an isotype-matched control mAb on ice, and the binding of test mAbs was detected with a $F(ab')_2$ fluorescein-isothiocyanate-conjugated goat anti-rat IgG (Serotec, Oxford, UK).

Samples were analysed using an EPICS XL flow cytometer (Beckman Coulter, Buckinghamshire, UK). For confocal microscopy, permeabilized (0.5% Triton X-100) or non-permeabilized cell samples were incubated with GoH3 with or without a rabbit anti-mouse-NE polyclonal Ab (gift from J. Roes, University College London, London, UK) or appropriate control antibodies at RT for 1 hour. Binding of primary mAbs was detected using goat anti-rat Alexa-Fluor-488- and/or goat anti-rabbit Alexa-Fluor-633-conjugated secondary antibodies (Molecule Probes, Eugene, USA). Staining was analysed using a Zeiss LSM 5 Pascal confocal microscope.

Quantification of NE activity on the cell surface of transmigrated peritoneal neutrophils

Mice were injected i.p. with 1 ml sterile saline or 10 ng IL-1β and, after 4 hours, killed by CO_2 asphyxiation. Peritoneal cavities were lavaged using ice-cold PBS (supplemented with 0.25% BSA and 2 mM EDTA) for isolation of peritoneal neutrophils. Alternatively, blood neutrophils were prepared as described above. Cell suspensions were added to 96-well plates (coated overnight at 4°C with 1 μg ml^{-1} BSA and blocked with 1% heat-inactivated BSA) such that each well contained a total of 4×10^5 neutrophils. Following centrifugation, cells were fixed with 3% PF plus 0.5% glutaraldehyde and then washed to remove fixative before assay of NE activity using a fluorogenic substrate specific for elastase, methoxysuccinyl-Ala-Ala-Pro-Val-7-amino-4-trifluoromethyl coumarin (MeOSuc-Ala-Ala-Pro-Val-AFC; Enzyme Systems Products, Livermore, USA), as previously described (Owen et al., 1997). A standard curve using purified human NE was established in each experiment and used to represent the results in terms of murine enzyme activity equivalent to the activity detected from ng ml^{-1} of purified human NE (Young et al., 2004).

In vitro neutrophil transmigration assay

Polycarbonate filters (3 μm pore size) of 96-well NeuroProbe chemotaxis chambers (Neuro Probe, Gaithersburg, MD) were first coated with laminin 1 (15 μg ml^{-1}; Invitrogen, Paisley, UK) at 4°C overnight and then further coated with saline or a combination of 20% PECAM-1, 80% ICAM-1 (the total concentration of the solution added to the plates being 10 μg ml^{-1}) at RT for 2 hours. Control filters were coated with 2% BSA. Bone-marrow neutrophils (2×10^5 cells), untreated or treated with the NE inhibitor ONO-5046 (50 μM final concentration), the mAb GAME 46 against β2 integrins (10 μg ml^{-1}), the mAb GoH3 (10 μg ml^{-1}) or relevant control mAbs, suspended in PBS supplemented with 10 mM HEPES, 10 mM glucose and 0.25% BSA, were placed in the top compartments of the chemotaxis chambers. The bottom wells contained saline or 10^{-7} M IL-8 and all tests were run in triplicate. The chambers were then incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 3 hours, after which the number of cells in the bottom wells was counted by microscopy after Kimura staining.

Statistics

All results are expressed as means±s.e.m. Statistical significance was assessed by one-way ANOVA with the Neuman-Keuls multiple-comparison test. Where two variables were analysed, Student's *t*-test was used. $P<0.05$ was considered to be significant.

Results

Both α6 integrins and NE are required for neutrophil transmigration through mouse cremasteric venules in vivo

Previous studies from our group have shown that transmigrated peritoneal neutrophils express higher levels of α6β1 on their cell surface than blood neutrophils (Dangerfield et al., 2002). In agreement with these observations, we have now found that IL-1β elicited transmigrated neutrophils in the cremaster muscle exhibit high levels of α6 on their cell surface as detected by immunofluorescence staining (using the mAb GoH3) and analysed by confocal microscopy (Fig. 1).

Our previous studies also demonstrated that α6-integrin blockade results in suppression of neutrophil migration through the PBM (Dangerfield et al., 2002), and we have now extended these findings by investigating the potential co-operative interaction of these integrins with the neutrophil serine protease NE. For this purpose, the roles of α6 integrins

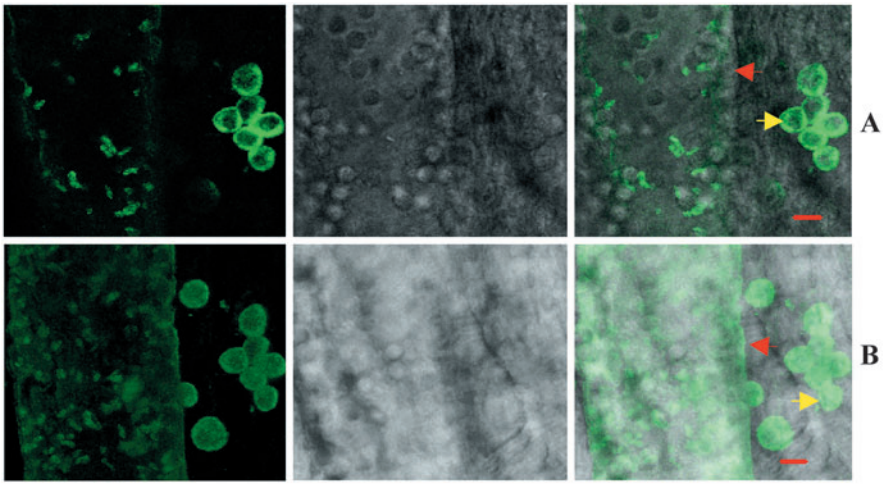

Fig. 1. Transmigrated leukocytes express α6 integrins on their cell surface in IL-1β-stimulated mouse cremaster muscles, as observed by confocal microscopy. Mouse cremaster muscles were injected i.s. with IL-1β (30 ng) and, 4 hours later, the tissues were dissected away from the animals, fixed in 4% PF and incubated with the primary mAb against α6 integrins, GoH3. Binding of GoH3 was detected using a secondary antibody conjugated to Alexa Fluor 488. Samples were observed at RT using a Zeiss LSM 5 Pascal confocal laser-scanning microscope (using a 40× water-dipping achroplan objective with a numerical aperture of 0.75) equipped with an argon laser (excitation wavelength of 488 nm). Typically, multiple optical sections of tissue samples, running through the whole depth of the tissue, were captured and imaged using the software's automatic scanning mode. In selected experiments, *z*-axis stack images (collected at every 0.8-1.2 μm depth) were saved and used for three-dimensional reconstruction analysis using the LSM 5 Pascal software (version 3.2). The images show expression of α6 integrins on leukocytes in the extravascular tissue, displayed as fluorescence (left), differential-interference contrast (DIC) (middle) and merged (right) patterns. (A) This series was obtained from the mid-level section of the marked leukocyte (yellow arrow), demonstrating a pronounced ring-like expression profile, indicating cell-surface expression. (B) A three-dimensional reconstructed image of the same tissue shown in two dimensions in A, illustrating the more global-like distribution of α6 integrins on transmigrated leukocytes. Leukocytes and vessel walls are shown by yellow and red arrows, respectively. Scale bars, 10 μm.

and NE in leukocyte transmigration through IL-1β-stimulated cremasteric venules were investigated by intravital microscopy using the mAb GoH3 against α6 integrins and the specific NE inhibitor ONO-5046, respectively. As part of these studies, the specificity of ONO-5046 was validated by using NE-deficient mice.

GoH3 (3 mg kg^{-1}, i.v. bolus) or ONO-5046 (50 mg kg^{-1}, i.v. bolus followed by infusion of 50 mg kg^{-1} $hour^{-1}$) inhibited neutrophil transmigration (57% and 39%, respectively) but not adhesion within IL-1β-stimulated cremasteric venules of WT mice (Fig. 2A,B). Furthermore, the combined administration of both blockers led to a greater level of inhibition (77%). In order to determine the stage of leukocyte transmigration at which these inhibitory effects were occurring, selected tissues were further analysed by confocal microscopy. For this purpose, tissues were immunofluorescently stained with Abs against PECAM-1 (as a marker for endothelial cells), laminin 10 (as a marker for venular basement membrane) and CD11b (as a marker for leukocytes) before being observed using a confocal microscope. Analysis of 1 μm optical sections running through whole intact venules from at least nine random sections per tissue indicated that there were more leukocytes trapped within venular walls (i.e. between endothelial cells and the PBM) in tissues from animals treated with ONO-5046 or GoH3 alone (1.5- and 2.8-times increases compared with tissues from IgG-and-saline-injected mice; n=3-4 mice). Interestingly, the extent of leukocytes trapped within venular walls was most pronounced in tissues from mice treated with both ONO-5046 and GoH3 (8.4-times increase, P<0.05; Fig. 2C-E). Crucially, whereas ONO-5046 suppressed neutrophil transmigration in WT mice, it had no effect in NE-deficient animals, indicating that the compound is a specific NE inhibitor. Furthermore, the normal level of neutrophil transmigration in response to IL-1β in NE KO mice compared with WT mice [in agreement with our previous findings (Young et al., 2004)] and the apparently conflicting results

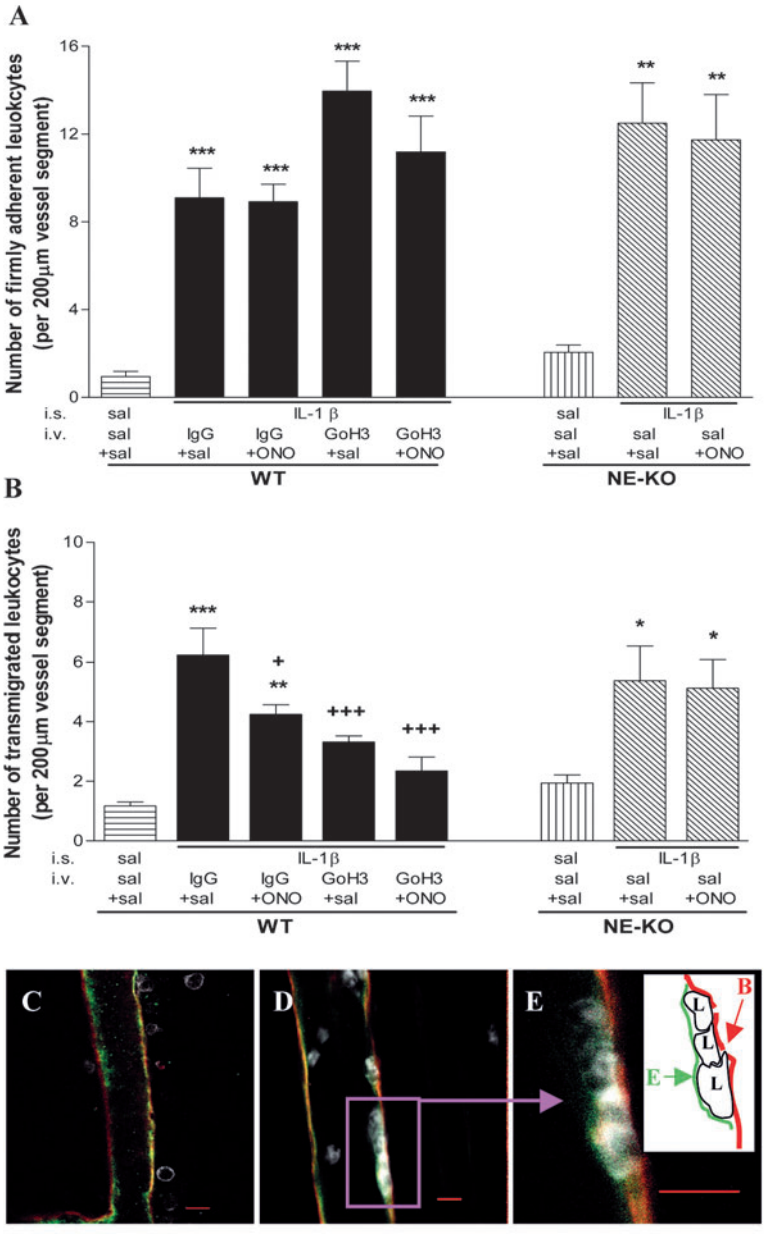

Fig. 2. The mAb GoH3 against α6 integrin and the NE inhibitor ONO-5046 suppress leukocyte transmigration through IL-1β-stimulated cremasteric venules in vivo. (A,B) WT or NE KO mice were injected i.v. with saline, control IgG or mAb GoH3 (both at 3 mg kg^{-1}) 15 minutes before i.s. injection of saline or IL-1β (30 ng). 2 hours later, the jugular vein was cannulated for infusion of either saline or the NE inhibitor ONO-5046 (50 mg kg^{-1} bolus followed by continuous infusion of 50 mg kg^{-1} $hour^{-1}$ using a syringe pump), the cremaster muscle was exteriorized and leukocyte responses quantified by intravital microscopy. (A,B) Leukocyte responses of firm adhesion and transmigration at 4 hours after IL-1β administration in the different groups of animals. The data shown are the means±s.e.m. from samples of 4-12 mice per group. Statistically significant differences in responses between mice receiving i.s. saline and mice receiving i.s. IL-1β are shown by asterisks: $^{**}P$<0.01; $^{***}P$<0.001. In mice receiving i.s. IL-1β, differences to animals injected with i.v. IgG-saline (control group) are shown by crosses: ^{+}P<0.05; ^{+++}P<0.001. (C-E) IL-1β-stimulated cremaster muscles from mice injected with either i.v. IgG-saline (C) or GoH3/ONO-5046 (D,E) were immunostained with specific mAbs for expression of PECAM-1 (APC; green), laminin 10 (Alexa Fluor 555; red) and CD11b (Alexa Fluor 488; white). Samples were observed at RT using a Zeiss LSM 5 Pascal confocal laser-scanning microscope (using a 40× water-dipping achroplan objective with a numerical aperture of 0.75) equipped with argon (excitation wavelength of 488 nm) and helium-neon (excitation wavelengths of 543 nm and 633 nm) lasers; the figure shows merged images captured from the three channels used. (E) A high-magnification image of the specified vessel segment shown in D in order to illustrate better the localization of leukocytes within the vessel wall. This is further indicated with an accompanying schematic diagram in which the position of endothelial cells (as identified using an anti-PECAM-1 mAb) is shown by a green line, the position of the basement membrane (as identified by an anti-laminin-10 Ab) is shown by a red line and trapped leukocytes (identified using an anti-CD11b mAb) are shown in white. All images are from tissue samples stained and analysed blind with at least nine random fields per tissue being captured from three or four mice in each group. Bars, 10 μm.

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obtained with the NE inhibitor are strongly indicative of developmental compensatory mechanisms in the genetically modified animals.

PECAM-1 ligation on mouse neutrophils increases cell-surface expression of α6 integrins but not NE

To investigate the molecular interactions by which α6 integrins and NE might be used by transmigrating neutrophils, the role of PECAM-1 ligation in upregulation of these molecules was investigated using an in vitro model. Purified mouse blood neutrophils were incubated for 30 minutes at RT in 96-well plates coated with BSA (control) or different combinations of PECAM-1, ICAM-1 and IL-8 before being immunostained with the GoH3 mAb or an anti-NE Ab and analysed by flow cytometry and/or confocal microscopy. In designing these experiments, we hypothesised that, in order to achieve a significant PECAM-1-dependent signalling response, the experimental conditions should promote a significant adhesive interaction between the neutrophil and the protein-coated plates. As a result, because PECAM-1 by itself is not an efficient adhesive substrate for neutrophils (data not shown), certain plates were coated with ICAM-1 as well as IL-8, to achieve optimal adhesion and hence contact between the cells and PECAM-1; these adhesive conditions might also be closer to those encountered by leukocytes in stimulated venules in vivo.

Fig. 3A-D shows expression of α6 integrins on neutrophils

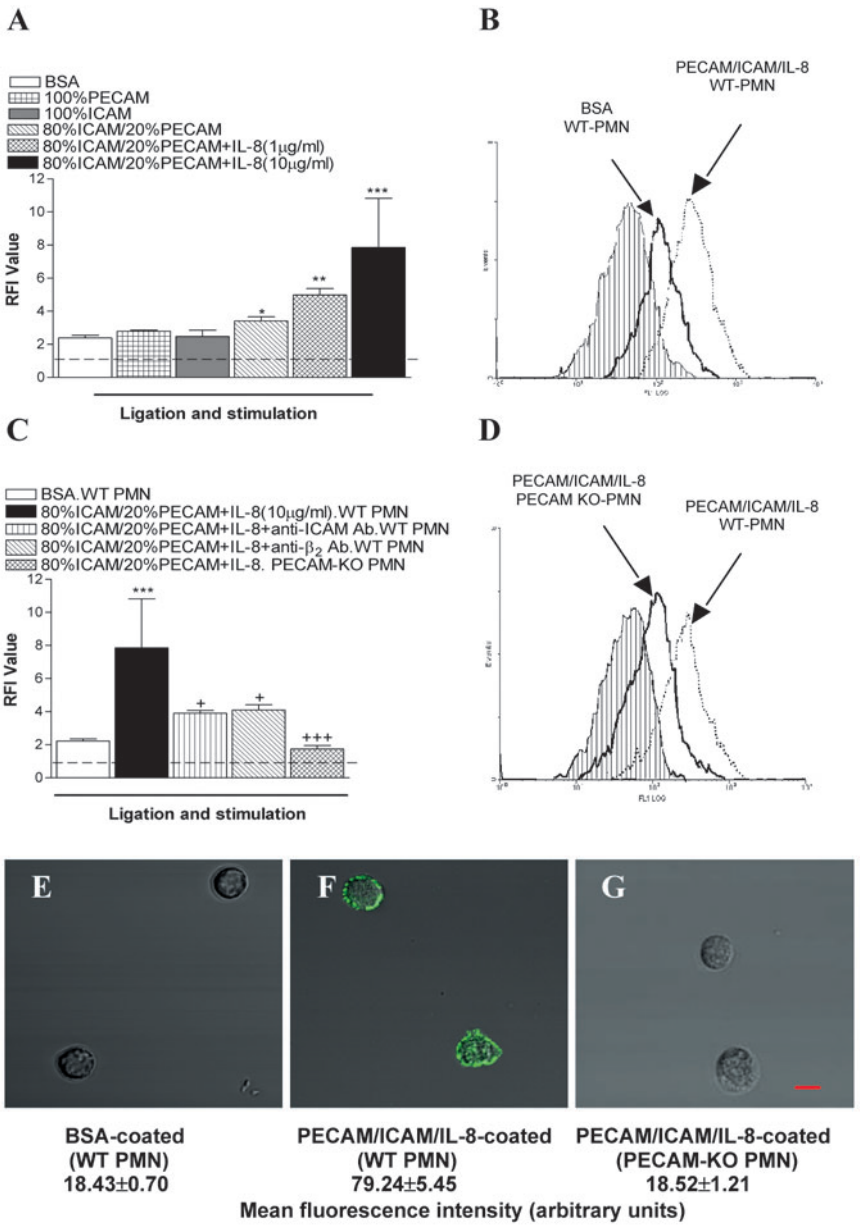

Fig. 3. PECAM-1 ligation enhances cell-surface expression of α6 integrins on mouse blood neutrophils in vitro as quantified by flow cytometry and observed by confocal microscopy. Blood-derived neutrophils incubated for 30 minutes in wells of 96-well plates, coated with BSA (control) or different combinations of PECAM-1, ICAM-1 and IL-8, were assayed for α6 integrins expression by flow cytometry. The binding of the primary mAb GoH3, relative to the binding of an isotype-matched control mAb, is expressed in terms of relative fluorescence intensity (RFI). (A) Cell-surface expression of α6 integrins on WT blood neutrophils after adhesion to different proteins and protein combinations. (C) Cell-surface expressions of α6 integrins on WT neutrophils incubated in wells in the presence or absence of anti-ICAM-1 or anti-β2 mAbs, and expression on the cell surface of PECAM-1-deficient neutrophils. Results represent means±s.e.m. from three to six samples in each condition using different neutrophil preparations. Asterisks and crosses indicate significant differences from samples incubated in BSA-coated wells: $*P<0.05$; $**P<0.01$; $***P<0.001$ (A,C), and significant differences from samples incubated in wells coated with PECAM-1, ICAM-1 and IL-8 (total protein being 10 μg ml^{-1}): $^{+}P<0.05$; $^{+++}P<0.001$ (C). (B,D) Representative histograms from experiments shown in A,C, with the hatched histograms representing binding of control IgG. Selected samples were also analysed using a confocal laser-scanning microscope (Zeiss LSM 5 Pascal incorporating a 100× oil A-plan objective with a numerical aperture of 1.25) equipped with an argon laser (excitation wavelength of 488 nm). Expression of α6 integrins was indirectly observed using the mAb GoH3 against α6 integrins and an Alexa-Fluor-488-conjugated secondary antibody. Representative images of mid-level sections of cells from different conditions are shown. (E) WT neutrophils incubated in BSA-coated wells. (F) WT neutrophils incubated in PECAM-1/ICAM-1/IL-8 (10 μg ml^{-1})-coated wells. (G) PECAM KO neutrophils incubated in PECAM-1/ICAM-1/IL-8 (10 μg ml^{-1})-coated wells. The binding of the anti-α6-integrin mAb under different experimental conditions, corrected for the binding of an isotype-matched control mAb and expressed in terms of mean fluorescence intensity (arbitrary units), is also shown (mean±s.e.m. from three to six samples in each condition using at least three different neutrophil preparations). Bar, 10 μm.

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incubated in different protein-coated wells, as quantified by flow cytometry. Incubation of cells in wells coated with PECAM-1, ICAM-1 or IL-8 alone or with both ICAM-1 and IL-8 did not lead to a significant change in cell surface expression of α6 integrins compared with neutrophils incubated in BSA-coated wells (Fig. 3A and data not shown). However, coating of wells with both PECAM-1 and ICAM-1 led to a small but significant increase in expression of α6 integrins (Fig. 3A). This response was further increased in the presence of increasing concentrations of IL-8 (Fig. 3A,B). Whereas Abs against ICAM-1 or β2 integrins partially suppressed this response, clear evidence for an absolute requirement for PECAM-1 ligation in increased expression of α6 integrins was provided by experiments involving neutrophils from PECAM-1 KO mice (Fig. 3C,D). The samples analysed by flow cytometry were also directly observed by confocal microscopy. In agreement with the flow cytometry results, WT neutrophils incubated in wells coated with the combination of PECAM-1, ICAM-1 and IL-8 (Fig. 3F) exhibited higher cell-surface expression of α6 integrins than cells incubated in BSA-coated wells (Fig. 3E), a response that was not observed with PECAM-1 KO neutrophils (Fig. 3G). These results yet again indicate that mobilization of neutrophil intracellular α6 integrins to the cell surface is PECAM-1 dependent.

To investigate the role of PECAM-1 in mobilization of NE to the cell surface of neutrophils, cells (WT or PECAM-1 KO) were incubated in wells of 96-well plates coated with either BSA or the combination of PECAM-1, ICAM-1 and IL-8 before being permeabilized, immunostained for both NE and α6 integrins, and analysed by confocal microscopy. Observation of neutrophils incubated in BSA-coated wells confirmed the presence of both molecules within intracellular stores although they did not appear to be co-localized (Fig. 4A). Analysis of WT neutrophils incubated in wells coated with a combination of PECAM-1, ICAM-1 and IL-8 showed clear evidence for the mobilization of NE and α6 integrins to the cell surface, as indicated by the ring-like expression profile of the molecules in 88.9% and 87.8% of neutrophils analysed, respectively (Fig. 4A). By contrast, under the same incubation conditions, a significantly lower proportion of PECAM-1 KO neutrophils exhibited cell-surface expression of α6 integrins

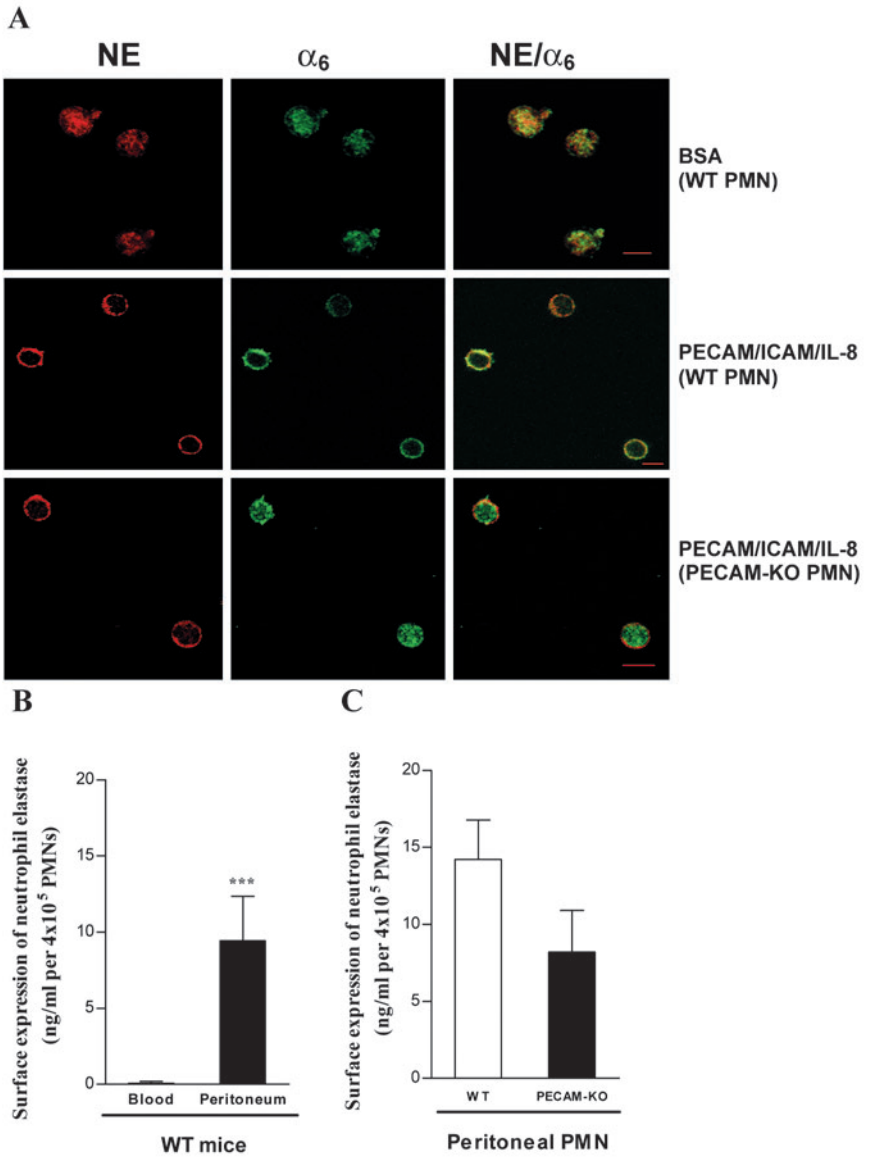

Fig. 4. Cell-surface expression of NE on mouse blood neutrophils in vitro and in vivo occurs via PECAM-1-independent mechanisms. (A) Blood neutrophils from WT or PECAM KO mice were incubated for 30 minutes in 96-well plates coated with BSA or the combination of PECAM-1, ICAM-1 and IL-8 (total protein being 10 μg ml^{-1}) before being permeabilized and immunostained for expressions of NE (red; Alexa Fluor 633) or α6 integrins (green; Alexa Fluor 488), and analysed at RT using a Zeiss LSM 5 Pascal confocal laser-scanning microscope (using a 100× oil A-plan objective with a numerical aperture of 1.25) equipped with HeNe (excitation wavelength of 633 nm) and argon (excitation wavelength of 488 nm) lasers. Images are shown of mid-level sections of representative cells from three independent experiments. Bar, 10 μm. (B,C) Cell-surface expression of NE was quantified on blood or IL-1β-elicited peritoneal neutrophils using a fluorogenic substrate. Briefly, mice (WT or PECAM-1 KO) were injected i.p. with saline (1 ml) or IL-1β (10 ng) and, 4 hours later, killed by CO_2 asphyxiation. Purified blood neutrophils or peritoneal leukocytes were added to 96-well plates such that each well contained a total of 4×10^5 neutrophils. After centrifugation, the samples were fixed and assayed for NE activity. (B) A comparison between NE activity on the cell surface of WT blood and peritoneal neutrophils (significant statistical difference between samples is indicated by asterisks: ***P<0.001). (C) A comparison between NE activity on the cell surface of IL-1β-induced transmigrated neutrophils in WT and PECAM-1 KO mice. Results represent the means±s.e.m. of samples obtained from seven to ten mice in each group.

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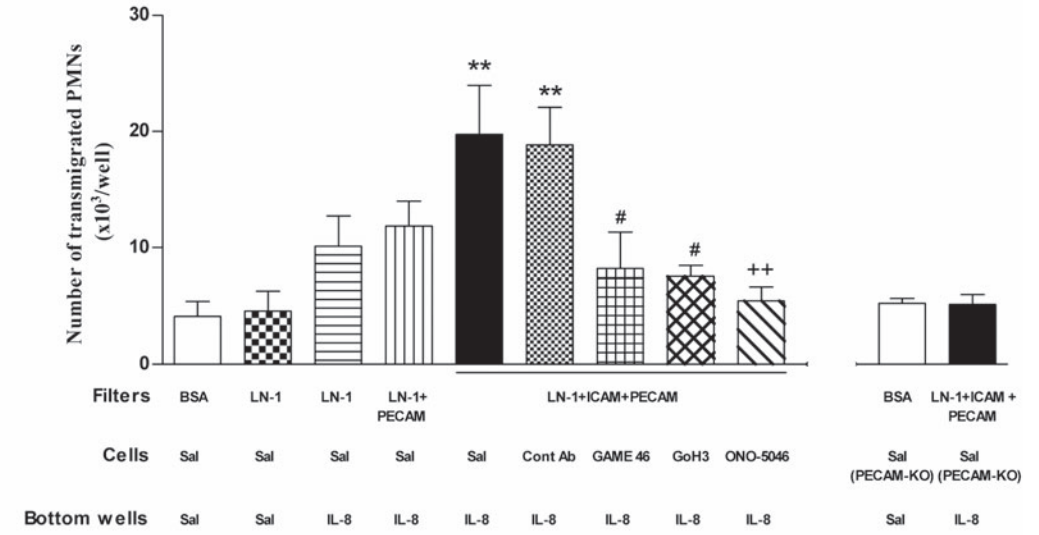

Fig. 5. Migration of murine neutrophils through laminin-coated filters is dependent on PECAM-1, α6 integrins and NE. 96-well NeuroProbe chemotaxis chambers were used to investigate neutrophil migration through laminin-coated filters. Transmigration filters (3 μm pores) were coated with 2% BSA, laminin-1 (LN-1; 15 μg ml^{-1}) or laminin-1 coated with a combination of 20% PECAM-1 and 80% ICAM-1 (using a solution with a total protein concentration of 10 μg ml^{-1}). Bone-marrow-derived mouse neutrophils (2×10^5) obtained from wild-type or PECAM-1 KO mice were placed on top of the filters with saline or IL-8 (10^{-7} M) in the bottom wells. Before addition to filters, cell samples were treated with saline (control), control IgG1b or IgG2a mAbs (both at 10 μg ml^{-1}; these data were pooled because there was no significant difference between them), a mAb against mouse β2 integrins (10 μg ml^{-1}), the mAb GoH3 against α6 integrin (10 μg ml^{-1}) or the NE-specific inhibitor ONO-5046 (50 μM final concentration), as indicated. After an incubation period of 3 hours at 37°C, the numbers of transmigrated cells in the bottom wells were counted by microscopy after Kimura staining. All samples were run in triplicate and the results represent means±s.e.m. from three to five independent experiments. Statistically significant responses from transmigration through BSA-treated filters are shown by asterisks (**P<0.01). Significant inhibition of transmigration through filters coated with laminin-1/PECAM-1/ICAM-1 in samples treated with GAME 46 or GoH3 compared with control mAb-treated samples are shown by hashes ($^{\#}P$<0.05) and significant inhibition of response by the NE inhibitor ONO-5046 compared with saline-treated cells is shown by crosses (^{++}P<0.01).

(30.3%) while exhibiting normal cell-surface expression of NE (82.7%). For these quantifications, a total of 172 stained WT neutrophils and 120 stained PECAM-1 KO neutrophils were analysed from samples prepared from four mice, in 5-12 random fields of view.

The role of PECAM-1 in the regulation of cell-surface expression of NE on transmigrated neutrophils was also investigated in vivo. Using an IL-1β (10 ng) elicited peritonitis model (4 hour reaction), a significant increase in cell-surface activity of NE was detected on transmigrated neutrophils compared with blood neutrophils, in WT mice (Fig. 4B). This response was not, however, significantly different in PECAM-1 KO animals (Fig. 4C). Collectively, the present in vitro and in vivo results demonstrate that, in contrast to the findings with α6 integrins, mobilization of NE to the cell surface of mouse neutrophils is PECAM-1 independent.

Both α6 integrins and NE are required for neutrophil transmigration through laminin-coated filters

In the light of the above in vitro and in vivo findings, and given that both α6 integrins and NE have been shown to interact with laminin (Timpl, 1996; Sonnenberg et al., 1990; Heck et al., 1990; Steadman et al., 1993), a key component of the venular basement membrane, the role of α6 integrins and NE in neutrophil migration through laminin-coated filters in vitro was investigated in a final series of experiments. To overcome the problem of insufficient availability of mouse blood neutrophils, bone-marrow-derived neutrophils were used in these studies.

In the absence of a chemoattractant in the bottom well of the transmigration chambers, neutrophils did not exhibit an enhanced level of transmigration through laminin-1-coated filters compared with control BSA-coated filters (Fig. 5). This response was, however, modestly increased when IL-8 (10^{-7} M) was placed in the bottom well; also, whereas no significant difference in the extent of transmigration occurred when the filters were coated with laminin and PECAM-1 or with laminin and ICAM-1 (Fig. 5 and data not shown), a significant increase in transmigration was observed when the filters were co-coated with laminin, PECAM-1 and ICAM-1. This enhanced transmigration response was presumably as a result of increased expression of both α6 integrins and NE (Figs 3, 4), and, indeed, evidence for functional roles of these molecules was obtained in studies involving specific blockers. Hence, Fig. 5 shows that IL-8-induced neutrophil transmigration through filters coated with laminin, PECAM-1 and ICAM-1 was suppressed by the mAb GoH3 against α6 integrins (76.9% inhibition) and the specific NE inhibitor ONO-5046 (10-50 μM final concentration; 91.5% inhibition). Furthermore, the response was significantly suppressed by a mAb against β2 integrin (73% inhibition), an effect that appeared to be due largely to suppression of neutrophil adhesion, rather than to transmigration (data not shown). Of relevance, neutrophils isolated from PECAM-1-deficient mice failed to exhibit a significant transmigration response through laminin-1/PECAM-1/ICAM-1-coated filters compared with control BSA-coated filters. Collectively, these data suggest that, for conditions under which α6 integrins and NE are expressed on the cell surface of neutrophils (i.e. when in contact with PECAM-1 and ICAM-1, respectively), both molecules can mediate neutrophil migration through laminin.

Discussion

Emigration of neutrophils from the intravascular

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compartment to the extravascular tissue is a central event in both innate and adaptive immunity and inflammation. The final barrier that leukocytes must traverse in this process is the PBM, a dynamic and self-assembled network of proteins, glycoproteins and proteoglycans that surrounds the vascular endothelium and pericytes. The mechanism by which neutrophils breach this barrier are poorly understood, although its heterogeneous nature (composed of molecules such as laminins, collagen type IV and entactin) suggests that this barrier is penetrated by an elaborate mechanism involving both adhesive and possibly proteolytic events. With respect to the former, we have previously provided evidence for the involvement of the integrin α6β1 in neutrophil migration through the PBM of IL-1β-stimulated venules in vivo, a mechanism that was associated with upregulation of integrin α6β1 on peritoneal neutrophils in a PECAM-1-dependent manner (Dangerfield et al., 2002). To extend these observations, we have now directly compared the roles of α6 integrins and the neutrophil serine protease NE in neutrophil transmigration, and have investigated the role of PECAM-1 in regulating the expression of these molecules. Collectively, the findings indicate that, although both α6 integrins and NE mediate neutrophil migration through the PBM in vivo, the adhesive mechanisms that regulate their cell-surface expression are different (i.e. PECAM-1 dependent and independent, respectively).

The integrin α6β1 is expressed by many cell types (Sonnenberg et al., 1988; Sonnenberg et al., 1987; Bohnsack, 1992; Terpe et al., 1994) including neutrophils, in which it is considered to be the major receptor for laminin. Studies in which mAbs against α6 integrins have blocked neutrophil migration through endothelial-cell monolayers cultured on laminin (Kitayama et al., 2000) or inhibited neutrophil migration through IL-1β-stimulated venules at the level of the PBM (Dangerfield et al., 2002) have strongly implicated integrin α6β1 in the process of leukocyte transmigration through the basement membrane. Another molecule implicated in this response is NE (Delclaux et al., 1996; Lee and Downey, 2001), a serine protease that has been shown to be mobilized to the leading edge of neutrophils migrating through cultured endothelial cells (Cepinskas et al., 1999). The role of NE in neutrophil migration, however, remains contentious (Huber and Weiss, 1989; Lee and Downey, 2001). Because the molecular architecture of the PBM suggests that a proteolysis step might facilitate the migration of leukocytes through this barrier, we sought to re-address this critical issue by investigating the functional roles and potential co-operative interaction of α6 integrins and NE in neutrophil transmigration.

The roles of α6 integrins and NE in neutrophil transmigration were investigated in IL-1β-stimulated cremaster muscles using the technique of intravital microscopy. In agreement with our previous findings using a peritonitis model (Dangerfield et al., 2002), transmigrated neutrophils in the cremaster muscle expressed high levels of α6 integrins as detected by immunofluorescence staining and analysis by confocal microscopy. Furthermore, both the mAb GoH3 against α6 integrins and the specific NE inhibitor ONO-5046, when administered alone or in combination, suppressed neutrophil transmigration (but not adhesion) through IL-1β-stimulated cremasteric venules. Immunofluorescence staining of tissues with markers for endothelium, venular laminin and leukocytes, and analysis by confocal microscopy indicated that these inhibitory effects occurred at least in part at the level of the PBM. Interestingly, however, the combined administration of the NE inhibitor and mAb GoH3 appeared to induce more leukocyte trapping within the vessel wall (i.e. inhibition of leukocyte migration through the PBM) than was observed with either reagent alone. This apparent synergistic effect of ONO-5046 and GoH3 suggests that NE might facilitate integrin α6β1 to mediate leukocyte transmigration through the PBM. Exactly how this could be achieved is currently unknown but it is conceptually possible that, during neutrophil transmigration, cell-associated NE facilitates the interaction of neutrophil α6β1 integrin with key binding sites within the laminin structure embedded in the PBM network. Importantly, the NE inhibitor ONO-5046 had no effect on leukocyte transmigration in NE KO animals, indicating the specificity of this compound as an NE inhibitor. Furthermore, the normal neutrophil infiltration observed in mice lacking NE, in agreement with previous reports (Belaaouaj et al., 1998; Tkalcevic et al., 2000; Young et al., 2004), and the discrepancy between these results and those in mice treated with the NE inhibitor are highly suggestive of developmental redundancy in the genetically modified animals. Although the nature of these NE-independent mechanisms is currently unknown, NE-deficient mice have (as part of our investigations) served the important purpose of demonstrating the selectivity of an NE inhibitor and hence, for the first time, provide direct evidence for the occurrence of developmental compensation in NE KO mice.

We have previously found evidence for an association between PECAM-1 and increased expression of α6 integrins on transmigrating neutrophils (Dangerfield et al., 2002). To investigate the mechanism by which α6 integrins and NE might be used by transmigrating neutrophils in vivo, the role of PECAM-1 ligation in regulation of cell surface expression of these molecules was directly investigated. For this purpose, an in vitro model was used in which purified murine blood neutrophils were incubated in 96-well plates coated with PECAM-1 (as well as ICAM-1 and, in some experiments, IL-8, in order to promote optimal adhesion and hence interaction between PECAM-1 on the neutrophil and the immobilized PECAM-1 in the wells). This procedure significantly enhanced cell-surface expression of α6 integrins compared with cells incubated in BSA-coated wells. Expression of α6 integrins on neutrophils incubated in PECAM-1/ICAM-1/IL-8-coated wells was significantly reduced in the presence of anti-ICAM-1 or anti-β2-integrin mAbs (~55%), and was totally inhibited when the experiment was performed using PECAM-1 KO neutrophils. Collectively, the data demonstrate that ligation of murine blood neutrophil PECAM-1 leads to increased cell-surface expression of α6 integrins, a response that appears to require, or be facilitated by, an initial adhesion response, promoted in the present study via an IL-8-induced ICAM-1/β2-integrin interaction. These results support the hypothesis that, within endothelial cell junctions, interaction of endothelial cell PECAM-1 with PECAM-1 on transmigrating neutrophils leads to rapid upregulation of α6 integrins, thus aiding their passage across the PBM. In addition, the findings add α6β1 to the growing list of integrins whose expression is directly regulated by PECAM-1 ligation (Tanaka et al., 1992; Berman and

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Muller, 1995; Varon et al., 1998; Chiba et al., 1999; Newman, 1997).

The above assay was also used to investigate whether PECAM-1 ligation led to enhanced cell-surface expression of NE. WT neutrophils incubated in BSA-coated wells largely exhibited intracellular expression of α6 integrins and NE (not co-localized), whereas cells incubated in wells coated with PECAM-1/ICAM-1/IL-8 exhibited cell-surface expression of both molecules, as observed by confocal microscopy. Interestingly, however, under the same experimental conditions, whereas PECAM-1 KO neutrophils did not show increased cell-surface expression of α6 integrins, these cells did show normal cell-surface mobilization of NE. Evidence for PECAM-1-independent mechanisms regulating cell-surface expression of NE was also obtained from in vivo studies. Although no NE enzymatic activity was detected on the cell surface of blood neutrophils, a significant level of activity was detected on the cell surface of IL-1β-induced peritoneal neutrophils in both WT and PECAM-1-deficient mice, with no significant difference being detected between the two strains of animals. Together, the results demonstrate that α6β1 and NE are not stored in the same intracellular compartments in murine neutrophils and that their enhanced cell-surface expression is induced via different adhesive mechanisms (i.e. PECAM-1-dependent and -independent pathways, respectively). Although this study was not extended to determine the precise mechanisms by which NE is induced to the cell surface of murine neutrophils, the in vitro findings do suggest that, within the protocol used, this response might have been elicited via direct or indirect effects of surface-bound IL-8. Indeed, previous studies have shown that both IL-8 and cross-linking of neutrophil β2 integrins can stimulate neutrophils for NE release (Rainger et al., 1998; Walzog et al., 1994).

Although regulated by different mechanisms, the above in vitro and in vivo findings suggest that both α6β1 and NE might support neutrophil migration through the PBM. Indeed, using an in vitro model, evidence was obtained for the involvement of both α6 integrins and NE in IL-8-induced murine neutrophil migration through laminin-coated filters. Collectively, these results suggest that neutrophil migration through the laminin network within the PBM can be facilitated by increased expression of α6β1, achieved via ligation of PECAM-1 at endothelial-cell junctions, as well as by NE expression and/or release. The latter response might be elicited by endothelial-cell-bound chemoattractants such as IL-8, either directly or indirectly via ligation of neutrophil β2 integrins after neutrophil stimulation.

Hence, in summary, the findings of the present study indicate that, although the integrin α6β1 and the serine protease NE are mobilized to the cell surface of transmigrating neutrophils via different adhesive mechanisms, the two molecules might act in concert to promote efficient migration of neutrophils through the PBM. Further studies are currently under way to understand the nature of this potential interaction and hence to shed more light on our understanding of the mechanisms by which leukocytes penetrate the PBM.

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