Expression and Regulation of the Metalloproteinase ADAM-8 during Human Neutrophil Pathophysiological Activation and Its Catalytic Activity on L-Selectin Shedding[1]

Maria Gómez-Gaviro,[2]* Maria Domínguez-Luis,[2†] Javier Canchado,[‡] Jero Calafat,[‡] Hans Janssen,[§] Enrique Lara-Pezzi,[¶] Anne Fourie,[‖] Antonio Tugores,[#] Agustín Valenzuela-Fernández, Faustino Mollinedo,[‡] Francisco Sánchez-Madrid,* and Federico Díaz-González[3†]**

A disintegrin and metalloproteinase domain (ADAM) proteins are a family of transmembrane glycoproteins with heterogeneous expression profiles and proteolytic, cell-adhesion, -fusion, and -signaling properties. One of its members, ADAM-8, is expressed by several cell types including neurons, osteoclasts, and leukocytes and, although it has been implicated in osteoclastogenesis and neurodegenerative processes, little is known about its role in immune cells. In this study, we show that ADAM-8 is constitutively present both on the cell surface and in intracellular granules of human neutrophils. Upon in vitro neutrophil activation, ADAM-8 was mobilized from the granules to the plasma membrane, where it was released through a metalloproteinase-dependent shedding mechanism. Adhesion of resting neutrophils to human endothelial cells also led to up-regulation of ADAM-8 surface expression. Neutrophils isolated from the synovial fluid of patients with active rheumatoid arthritis expressed higher amounts of ADAM-8 than neutrophils isolated from peripheral blood and the concentration of soluble ADAM-8 in synovial fluid directly correlated with the degree of joint inflammation. Remarkably, the presence of ADAM-8 both on the cell surface and in suspension increased the ectodomain shedding of membrane-bound L-selectin in mammalian cells. All these data support a potential relevant role for ADAM-8 in the function of neutrophils during inflammatory response. *The Journal of Immunology,* **2007, 178: 8053–8063.**

The recruitment of leukocytes into the inflammatory foci is a cardinal event in the inflammatory response. This cell accumulation is preceded by a highly coordinated sequence of interactions between leukocytes and endothelial cells, a process termed adhesion cascade (1, 2). Neutrophils are, as part of the innate immune response against pathogens, the first immune cells to be recruited at inflammatory foci, exerting their function via the phagocytosis of foreign bodies, the releasing of several antimicrobial polypeptides stored in their intracellular granules (3), and the generation of oxygen-free radicals. Human neutrophils contain three major different types of granules: azurophilic or primary granules, specific or secondary granules, and gelatinase-rich or tertiary granules, as well as an apparently releasable organelle named secretory vesicle (3, 4). Specific and tertiary granules, together with secretory vesicles, constitute a reservoir of a wide array of plasma membrane proteins involved in the adhesion cascade, that are translocated to the cell surface in response to neutrophil activation. Azurophilic granules are mainly involved in the phagocytic and microbicidal function of neutrophils and thereby contain a large number of lytic enzymes (3, 4).

The effective regulation of neutrophil influx to tissue is important to minimize the destructive effect that neutrophil cytotoxic granule contents may produce in the normal tissue during the inflammatory response (5). Several adhesion molecules, including members of the selectin family, participate coordinately in the migration of flowing neutrophils through vascular endothelium (1, 2). Neutrophils use L-selectin to roll along endothelium in the initial phase of the adhesion cascade. In seconds, L-selectin is shed from the surface of rolling neutrophils and both rolling speed and neutrophil accumulation are increased when in vitro shedding is blocked (6). Thus, a correct regulation of the L-selectin ectodomain processing appears to be necessary for an efficient transendothelial migration of neutrophils (7). A member of the a disintegrin and metalloprotease domain (ADAM)[4] family of membrane

*Servicio de Inmunología, Hospital de la Princesa, Universidad Autónoma de Madrid, Madrid, Spain; †Servicio de Reumatología, Hospital Universitario de Canarias, La Laguna, Santa Cruz de Tenerife, Spain; ‡Centro de Investigación del Cáncer, Instituto de Biología Molecular y Celular del Cáncer, Consejo Superior de Investigaciones Científicas-Universidad de Salamanca, Salamanca, Spain; §Division of Cell Biology, The Netherlands Cancer Institute, Amsterdam, The Netherlands; ¶Unidad de Biología Molecular, Hospital de la Princesa, Madrid, Spain; ‖Johnson & Johnson Pharmaceutical Research and Development, San Diego, CA 92121; #Unidad de Investigación, Complejo Hospitalario Materno Insular de Gran Canaria, Las Palmas de Gran Canaria, Spain; and **Departamento de Medicina Física y Farmacología, Facultad de Medicina, Universidad de La Laguna, Tenerife, Spain

Received for publication June 19, 2006. Accepted for publication April 9, 2007.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by grants from Ministerio de Ciencia y Tecnología of Spain (SAF02/1634) and Fondo de Investigación Sanitaria (FIS04/1275) (to F.D.-G.) and from Fondo de Investigación Sanitaria (FIS04/0843, FIS01/1048), Fundación de Investigación Médica Mutua Madrileña, Fundación "La Caixa" (BM05-30-0), Junta de Castilla y León (CSI04A05) (to F.M.) F.D.-G. was a recipient of a grant for Research Intensification from Fondo de Investigaciones Sanitarias del Instituto de Salud Carlos III. M.G.-G.'s work was supported by a grant from Fondo de Investigaciones Sanitarias del Instituto de Salud Carlos III (BEFI 99/0259) and M.D.-L.'s work was supported by a grant from Fundación Rafael Clavijo and Fundación Española de Reumatología.

[2] M.G.-G. and M.D.-L. have contributed equally to this work.

[3] Address correspondence and reprint requests to Dr. Federico Díaz-González, Servicio de Reumatología, Hospital Universitario de Canarias, Ofra s/n. La Cuesta, 38320 La Laguna, Santa Cruz de Tenerife, Spain. E-mail address: jfdiaz@huc.canarias.org

[4] Abbreviations used in this paper: ADAM, a disintegrin and metalloproteinase domain; TACE, TNF-α-converting enzyme; OCL, osteoclast; RA, rheumatoid arthritis; sADAM, soluble form of ADAM; hADAM, human ADAM; MFI, mean fluorescence intensity; rMFI, relative MFI; sL-selectin, soluble L-selectin; MMP, matrix metalloproteinase.

metalloproteinases, ADAM-17 (CD156b), also known as TNF-α-converting enzyme (TACE), has shown proteolytic activity on L-selectin (8). Although it is well-established that ADAM-17 is required for efficient L-selectin shedding, the fact that TACE-deficient cells generate a significant shedding of L-selectin (9), in addition to other evidence (7), strongly suggests that there may be other sheddase and/or additional factors involved in this process.

The ADAM protein family consists of a number of widely distributed transmembrane glycoproteins that play key roles in sperm-egg binding and fusion, muscle cell fusion, neurogenesis, susceptibility to type I hypersensitivity, and proteolysis of several membrane proteins (10–12). The basic structure of an ADAM family protein is well-conserved and comprises a cysteine-rich domain, a disintegrin domain, with adhesive properties, and a metalloproteinase domain responsible for the ectodomain shedding of membrane proteins and for the cleavage of extracellular matrix components (11). Catalytically active ADAMs are usually activated by furin-catalyzed removal of the prodomain. However, some ADAM members do not seem to be catalyzed by furin-like proteases, including ADAM-8 (CD156a), whose prodomain is removed by a metalloproteinase (13). ADAM-8, originally cloned from monocytic cells (14), is expressed mainly in cells of the immune system, such as monocytes, granulocytes, and B cells. Although mice lacking the *ADAM-8* gene presents no major structural defects (15), different reports have suggested a role for ADAM-8 in a number of pathological processes (16, 17). Recently, ADAM-8 has been involved in osteoclast (OCL) fusion (18). Its expression is increased in OCLs compared with OCL precursors and stimulates OCL formation in murine bone marrow cultures. ADAM-8 has also shown to act as a sheddase. In particular, it is able to convert membrane-bound CD23 (19) and the neural cell adhesion molecule CHL1 into soluble forms (20). However, the regulation of the expression and/or function of ADAM-8 in cells of the immune system, where it is preferentially expressed, is still poorly understood. The recent implication of ADAM-8 in the allergic response (17), in addition to its potential role in the process of leukocyte infiltration shown by ADAM-8-transgenic mice (21) have increased the interest for this surface metalloproteinase in the regulation of the inflammatory response.

In this study, we describe the expression profile, localization, and regulation of ADAM-8 in human neutrophils. We show that ADAM-8 is localized both on the cell membrane and in the cytoplasmic granules of resting neutrophils. Upon cell activation, both in vitro and in vivo, ADAM-8 was mobilized from the granules to the cell membrane and finally released to the extracellular milieu by a metalloprotease-dependent proteolysis. The physiological relevance of these observations was supported by the finding that the presence of ADAM-8 in the knee joints of patients diagnosed with rheumatoid arthritis (RA) correlated with the intensity of the local inflammatory response. In addition, we show that both the membrane-bound and the active soluble form of ADAM-8 (sADAM-8) were able to cleave L-selectin from the plasma membrane, further supporting a key role for this metalloproteinase in neutrophil function at inflammatory sites.

Materials and Methods

Patients

Samples of synovial fluid were isolated from the knees of five patients with active RA, as defined by the American College of Rheumatology (22), and from five patients with a diagnosis of knee osteoarthritis. Peripheral blood was simultaneously obtained from the same patients. The Ethics Committee of Hospital de La Princesa approved the study and informed consent was obtained from all the patients.

Generation of anti-ADAM-8 mAbs

mAbs were generated as previously described (23). Briefly, BALB/c mice were immunized with the peptide CTMAHEMGHNLGMDHDENVQGC, containing the Zn^{2+}-binding consensus motif of the ADAM-8 catalytic domain. Hybridoma culture supernatants were tested by ELISA against the immunogenic peptides and yielded the anti-ADAM-8 mAb 2H5, an IgG2bκ. The specificity of this mAb is later shown in the results section.

Abs, reagents, and plasmids

Anti-human ADAM-8 (hADAM-8) mAb (clone 143338), a murine IgG1 recognizing amino acid 498–653 of human ADAM-8 ectodomain, was purchased from R&D Systems. All experiments involving flow cytometry or Western blot analysis for ADAM-8 were done with this mAb, except where indicated. The monoclonal Ig (IgG1) from the P3X63 myeloma cell line was used as negative control. Anti-L-selectin mAb Leu-8 was obtained from BD Biosciences. Anti-CD11b mAb Bear-1 has been previously described (24). Anti-human α-tubulin mAb was purchased from Sigma-Aldrich. The hydroxamic acid-based metalloproteinase inhibitor KD-IX-73-4 was a gift from Dr. T. K. Kishimoto (Boehringer Ingelheim Pharmaceuticals, Ridgefield, CT) (25). Recombinant human ectodomain of ADAM-8 (amino acid residues 1–497) was obtained from R&D Systems and used either inactive or activated with thermolysin (R&D Systems) following the manufacturer's recommendations. All other chemicals were purchased from Sigma-Aldrich. The expression vector pcDNA3 containing full-length ADAM-8 and its catalytically inactive form (H604A, H608A) has been previously described (19).

Cell isolation, growth, and treatments

Human neutrophils were freshly isolated from peripheral blood obtained from healthy donors by Ficoll-Hypaque (Pharmacia Diagnostics) density gradient centrifugation for 30 min at 400 × *g*, followed by erythrocyte sedimentation at 1 × *g* in 1.3% (w/v) dextran (Sigma-Aldrich) at room temperature for 20 min. The neutrophil-enriched fraction was further purified by hypotonic lysis of erythrocytes, yielding purity >95%. Isolation of human neutrophils from the synovial fluid obtained from the knees of RA patients was performed as previously described (26). Neutrophils were isolated from the blood of the same patients as described above. Experiments with neutrophils were conducted in 15 ml of disposable polypropylene tubes (Falcon Labware). Neutrophils were incubated in PBS alone or in the presence of 20 ng/ml human recombinant TNF-α (ImmunoTools) or 20 ng/ml PMA (Sigma-Aldrich) at either 4 or 37°C. HUVEC were obtained as previously described (23). Studies of neutrophils-HUVEC interaction were conducted in 24-well plates. HUVEC were grown until confluence and stimulated for 6 h with 20 ng/ml TNF-α or maintained in resting conditions. After washing five times with PBS, 1×10^6 resting neutrophils were placed in wells and allowed to adhere at 37°C under static conditions for 15 min. Then, wells were gently washed and nonadherent cells were pooled. Adherent cells were detached by trypsinization with trypsin-EDTA (Sigma-Aldrich). In the cell suspension obtained after trypsinization of the attached cells, neutrophils were discriminated from HUVEC by their side and forward scatter characteristics shown by flow cytometry. Adhered and nonadhered cells were labeled by indirect immunofluorescence for the detection of ADAM-8 and CD11b and analyzed with a FACScan flow cytometer (BD Biosciences), as described below. In the experiments where the metalloproteinase inhibitor was used, neutrophils were preincubated for 10 min at 37°C with 20 μM KD-IX-73-4, except when indicated.

HEK293 cells were grown in DMEM (PAA), 10% FCS, 50 IU/ml penicillin, 50 μg/ml streptomycin, and 2.5 μg/ml fungizone. CEM cells (acute lymphoblastic T cell leukemic cells) were maintained in Dulbecco's RPMI 1640 supplemented with 10% FBS, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. Jurkat cells were cultured in RPMI 1640 (PAA) supplemented with 10% FCS and 2×10^{-5} M 2-ME (Sigma-Aldrich). None of these three cell lines express ADAM-8 constitutively. All cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2. For transfections, HEK293 and CEM cells were nucleofected with 2 μg of total DNA by using VCA-1003 kit (Amaxa) according to the manufacturer's instructions. For the analysis of L-selectin shedding by ADAM-8 in HEK293 cells, 0.5 μg of pcDNA3-L-selectin plus 1.5 μg of pcDNA3-ADAM-8 either full-length or mutated were transfected and cell culture supernatants were collected at 48 h later for protein detection by an ELISA kit. CEM cells were transfected with 2 μg of pcDNA3-ADAM-8 either full-length or mutated and cell-free supernatant collected at 24 h for protein detection. We observed an important unspecific shedding of L-selectin induced by the transfection procedure itself in CEM cells. To avoid this effect, cells were incubated the first

20 h after transfection in medium containing 20 μM KD-IX-73-4. Then, the inhibitor was removed and cells were cultured in medium until supernatant was recovered 4 h later. Construct-expressing cells were assayed by flow cytometry at the indicated time.

ADAM-17-deficient ($DRM^{-/-}$) immortalized murine monocytes (27) stably expressing human L-selectin were generated by retroviral gene transfer using an established procedure (28). This cell line was cultured and expanded as described (29).

Flow cytometry analysis

Flow cytometry analysis was conducted essentially as described (24). Briefly, cells were treated as described above and then incubated with the different mAbs at 4°C for 30 min. After washing in PBS, cells were labeled with FITC-labeled goat anti-mouse Ig (DakoCytomation). At least 5×10^3 cells from each sample were analyzed in a FACScan flow cytometer (BD Biosciences) and data were collected in both linear and logarithmic scales. Mean fluorescence intensity (MFI) in linear scale was obtained adjusting the fluorescence gain so that ~5% of the cells of the sample with greatest fluorescence were positive in the highest fluorescence channel. The fluorescence produced by isotype matching control mAbs were considered as the background. In same experiments, because the fluorescence conditions were different from experiment to experiment, data were normalized to express the relative (rMFI), according to the following equation: $rMFI = (MFI_{stimulus} - MFI_{P3X63})/(MFI_{medium} - MFI_{P3X63})$.

Immunofluorescence microscopy

Immunofluorescence staining was performed on cells seeded on 18-mm circular glass coverslips (Fisher Scientific) in serum-free medium. After a 20-min incubation at 37°C for neutrophils and 90 min at 37°C for HEK293 cells, attached cells were fixed with 3.7% methanol-free formaldehyde (Polysciences) for 10 min at room temperature and, where indicated, were permeabilized with 0.2% Triton X-100 (Sigma-Aldrich) for 5 min at room temperature. To block nonspecific binding, coverslips were incubated with 10% normal goat serum (Sigma-Aldrich) for 1 h at room temperature, and then incubated for 30 min at 37°C with the primary Abs in an isotonic solution containing 10% normal goat serum. After washing five times with PBS and incubated for another 30 min at 37°C with a goat anti-mouse Alexa 488 conjugate (Molecular Probes), coverslips were washed and mounted in FITC-Guard mounting medium (Testog), and examined with either a Leitz Orthoplan microscope using a ×100 oil immersion objective or with a confocal microscope as indicated.

Immunoelectron microscopy

Resting human neutrophils and exudate neutrophils from skin window chambers after phagocytosis of latex beads were fixed for 24 h in 4% paraformaldehyde in 0.1 M PHEM buffer (60 mM PIPES, 25 mM HEPES, 2 mM $MgCl_2$, and 10 mM EGTA (pH 6.9)) and then processed for ultrathin cryosectioning as previously described (30). Cryosections (45 nm) were cut at −125°C using diamond knives (Drukker Cuijk) in an ultracryomicrotome (Leica Aktiengesellschaft) and transferred with a mixture of sucrose and methylcellulose onto formvar-coated copper grids (31). The grids were placed on 35-mm petri dishes containing 2% gelatin. For double immunolabeling, the procedure described by Slot et al. (32) was followed using 10- and 15-nm protein A-conjugated colloidal gold probes (EM Laboratory, Utrecht University, The Netherlands). After immunolabeling, the cryosections were embedded in a mixture of methylcellulose and uranyl acetate and examined with a Philips CM10 electron microscope. Negative controls were prepared by replacing the primary Abs by a nonrelevant rabbit or mouse Ab, showing no staining of the samples. The Abs used in double immunolabeling were: mouse mAb anti-ADAM-8 (R&D Systems); rabbit anti-human lactoferrin (Cappel Laboratories); rabbit anti-human myeloperoxidase from (DakoCytomation); rabbit anti-gelatinase (33), provided by Dr. N. Borregaard (Department of Hematology, National University Hospital, Copenhagen, Denmark); and rabbit anti-human albumin (Central Laboratory, Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands).

Subcellular fractionation

Resting or activated freshly prepared human neutrophils (~3–5 × 10^8) were gently disrupted and the postnuclear fractions were fractionated in 15%–40% (w/w) continuous sucrose gradients as described previously (34). Subcellular fractions were assayed for marker proteins specific for each organelle, namely lactate dehydrogenase (cytosol), HLA (plasma membrane), latent alkaline phosphatase (secretory vesicles), gelatinase (tertiary granules), lactoferrin (specific granules), and peroxidase (azurophilic granules) as described previously (34, 35). Secretory vesicles were not resolved from the plasma membrane under the fractionation conditions used (35). Membranes from each fraction were obtained by diluting the fractions with 50 mM Tris-HCl (pH 8.0), 100 mM NaCl, and through centrifugation at 45,000 rpm (100,000 × g) for 90 min at 4°C using a 70 Ti type rotor (Beckman Instruments). Finally, pellets were resuspended in 50 mM Tris-HCl (pH 7.5) containing 2 mM PMSF and stored at −20°C until use.

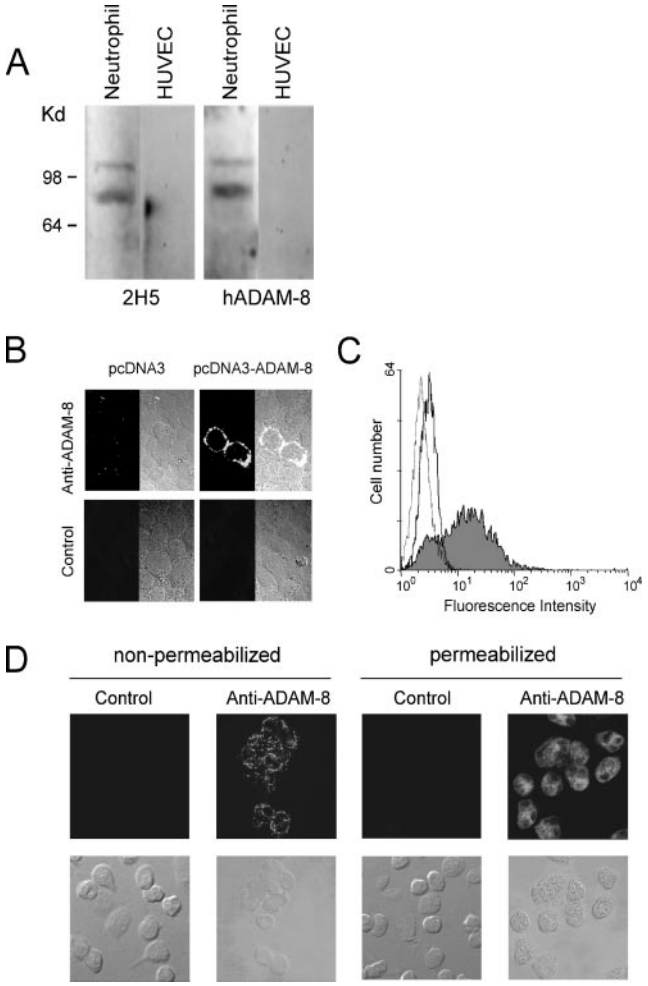

FIGURE 1. Expression of ADAM-8 in human cells. *A*, Western blot analysis of cell lysates from equivalent number of human neutrophils and HUVEC. Two mAbs to ADAM-8 were used: one raised against the catalytic domain peptide sequence of ADAM-8, 2H5 and another commercially available, hADAM-8 (see *Materials and Methods* for details). *B*, Immunofluorescence analysis of HEK293 cells transiently transfected with a full-length ADAM-8 cDNA or the empty expression vector. Following 48 h after transfection, cells were harvested, washed, and stained for surface expression of ADAM-8 using hADAM-8 mAb. *C*, ADAM-8 surface expression was monitored by flow cytometry using hADAM-8 mAb on J77 cells stably transfected with an ADAM-8 cDNA (shaded histogram) or the empty expression vector (unshaded histogram). Dotted histogram represents the negative control Ab (P3X63 myeloma). *D*, ADAM-8 distribution in nonpermeabilized and permeabilized human resting neutrophils. Cells were plated on uncoated crystal slides and treated with 2% formaldehyde in PBS for 5 min at 4°C. After fixation, some cells were permeabilized at 4°C with 0.5% Triton X-100 for 1 min, while others were maintained in PBS. Neutrophils were incubated with hADAM-8 mAb, followed by incubation with a goat anti-mouse Alexa 488 conjugate. After washing, cells from *B* and *D* were visualized by confocal fluorescence microscopy.

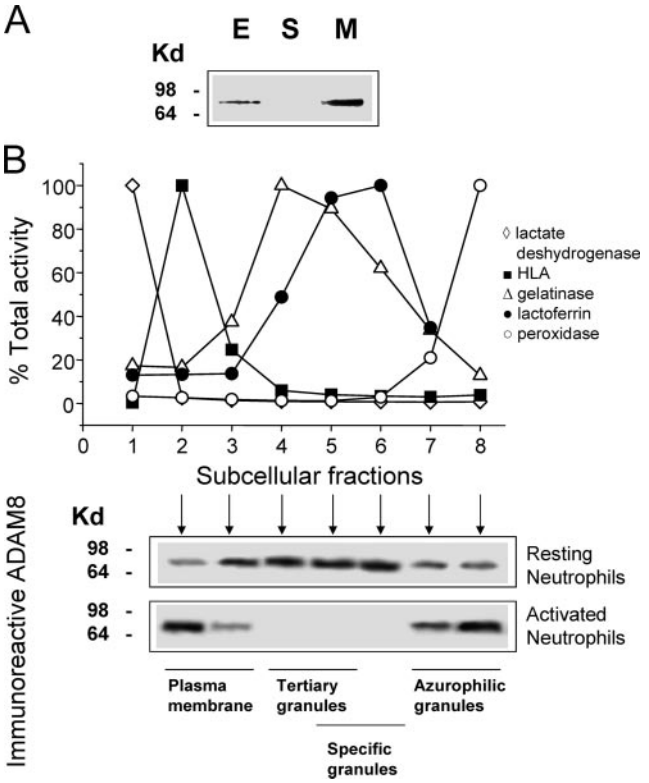

FIGURE 2. Subcellular distribution of ADAM-8 in human neutrophils. *A*, Equal amounts of postnuclear extract (E), soluble (S), and membrane (M) proteins (70 μg) from resting human neutrophils were run on SDS-PAGE and analyzed by immunoblotting. *B*, Enzymatic activity and immunoreactive ADAM-8 in subcellular fractions of neutrophils. Resting human neutrophils were gently disrupted and subjected to subcellular fractionation as described in *Materials and Methods*. Fractions (4-ml each, save fraction 1-cytosol-, 6 ml) were collected and analyzed for the activity of specific organelle markers, which are plotted normalized to the fraction with maximal activity. The following markers were assayed. Cytosol: lactate dehydrogenase (◊); plasma membrane: HLA (■); tertiary granules: gelatinase (△); specific granules: lactoferrin (●); azurophilic granules: peroxidase (○). Membrane proteins (50 μg) from the subcellular fractions 2–8 of resting and activated human neutrophils (1 μg/ml PMA, 10 min) were assayed for ADAM-8 by immunoblotting. Membranes from fractions enriched in plasma membrane, tertiary granules, specific granules, and azurophilic granules were analyzed. The molecular masses (K_d) of the protein markers are indicated on the *left.* All data shown are representative of three independent experiments.

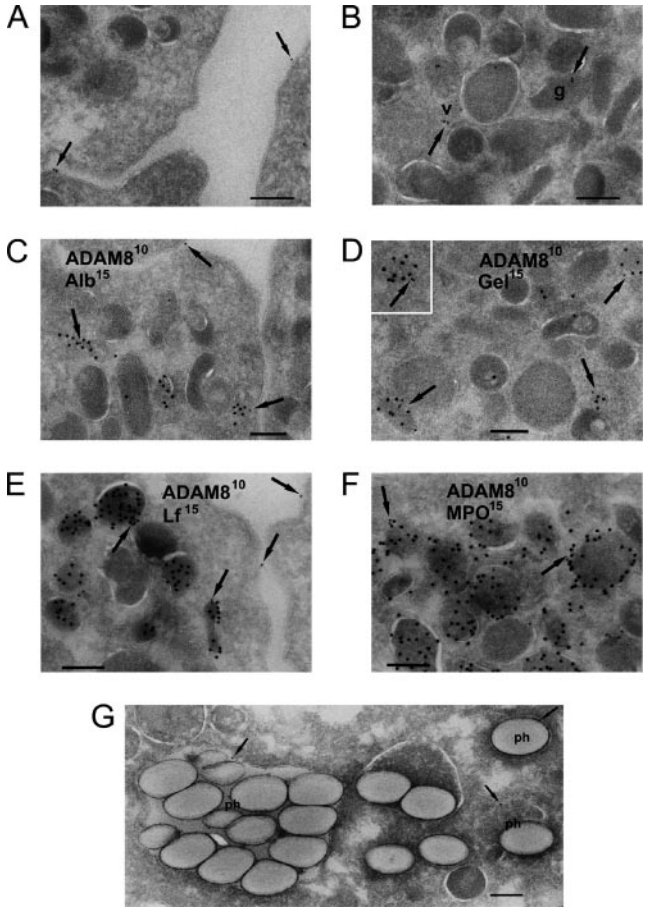

FIGURE 3. ADAM-8 is localized both at the plasma membrane and in the membranes of neutrophil intracellular granules. *A*, Electron microscopy images of resting neutrophils. Cryosections of neutrophils were immunogold labeled with mouse monoclonal anti-ADAM-8 Ab (10-nm gold). ADAM-8 labeling is detected at the plasma membrane as well as at the membrane of granules (g) and vesicles (v). *B*, ADAM-8 is labeled on the outer side of the plasma membrane facing the external medium and in the internal face of the membrane of granules and vesicles. Neutrophils were also double labeled with ADAM-8 and respectively with albumin (*C*), gelatinase (*D*), lactoferrin (*E*), or myeloperoxidase (MPO) (*F*) using specific immunogold-labeled Abs with different gold particle sizes (10 and 15 nm) as indicated. In all cases, some granules or albumin-enriched secretory vesicles, are double labeled (arrows). *Inset*, *D*, The presence of two gold particles of anti-ADAM-8 Ab in a gelatinase-positive granule. *G*, Localization of ADAM-8 in exudate neutrophils from skin window chambers after phagocytosis of latex beads. ADAM-8 (arrows) is localized in the phagolysosomes (ph). Bars, 200 nm.

Western blot assays

Equal amount of total proteins were separated by SDS-16% PAGE and immunoblotted as described previously (34). After blocking with 5% powdered defatted milk in TBST buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.05% Tween 20), blots were incubated overnight with 2H5 mAb (dilution 1/1000), anti-hADAM-8 mAb (dilution 1/1000), or anti-L-selectin mAb (dilution 1/1500) in TBST buffer. Ab reactivity was detected with a biotinylated anti-mouse IgG (diluted at 1/1000 in TBST buffer), followed by incubation with streptavidin-HRP conjugated (diluted 1/1000 in TBST buffer), using an ECL detection system (Amersham Biosciences).

Soluble L-selectin (sL-selectin) and ADAM-8 (sL-ADAM-8) ELISA

Neutrophils (7×10^6 cells/ml) freshly isolated from both peripheral blood and synovial fluid as described above, were incubated in PBS alone, with 20 ng/ml PMA or with 20 ng/ml TNF-α. After incubation at the appropriate temperatures for the times indicated, cells were eliminated by centrifugation (13,000 $\times$ *g* for 2 min at 4°C) and cell-free supernatants were collected and stored at −80°C. Synovial fluids obtained from the swollen knees of both RA patients and osteoarthritis patients were pelleted (300 $\times$ *g* at 4°C for 10 min) immediately after joint aspiration and cell-free supernatant was collected and stored. In control experiments, both neutrophil-free supernatants and cell-free synovial fluids were ultracentrifuged at 100,000 $\times$ *g* as described in subcellular fractionation, to discard subcellular fragments, including cell microparticles. Soluble ADAM-8 (sADAM-8) concentration was assessed by an ELISA kit (R&D Systems) following the manufacturer's instructions and the data were expressed as absolute concentration in picograms per milliliter or in relative units considering: 1) the concentration of ADAM-8 in the supernatant of neutrophils maintained at 4°C. sL-selectin from cell-free supernatants was detected by ELISA using an ELISA kit (Bender Medsystem and R&D Systems) following the manufacturer's instructions and data were expressed as relative concentration with respect to the basal amount of L-selectin in CEM cells and L-selectin-transfected HEK-293 cells, which was considered as 1.

Statistical analysis

Differences between groups were examined for statistical significance using the Wilcoxon's signed rank test. Values of $p < 0.05$ were considered significant.

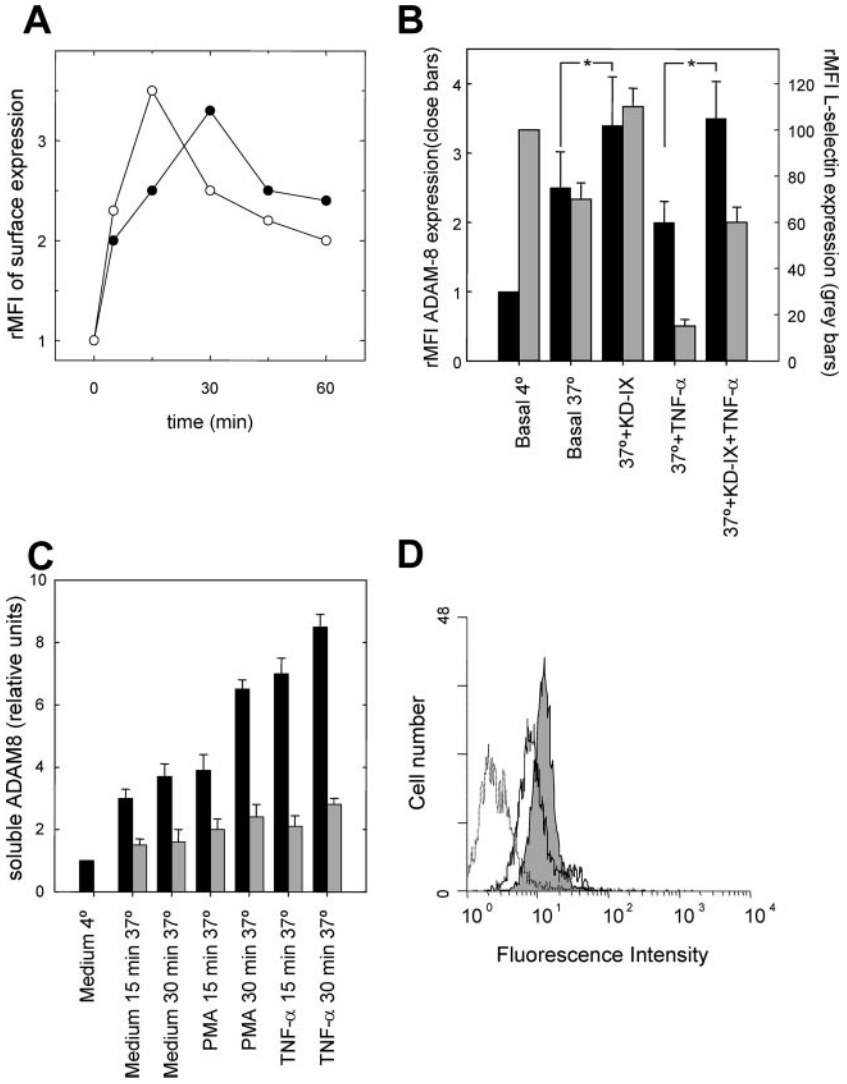

FIGURE 4. ADAM-8 is translocated to and released from the surface of neutrophils. *A*, Kinetics of ADAM-8 surface expression in neutrophils. Cells freshly isolated from peripheral blood were incubated for 5, 15, 30, 45, or 60 min, at 37°C, and the expression of ADAM-8 (○) and CD11b (●) was measured by flow cytometry. Data show one representative time-response experiment of five. *B*, Neutrophil surface expression of ADAM-8 and L-selectin. Neutrophils were preincubated for 10 min at 37°C, in the absence or presence of 20 μM of the hydroxamic acid-based inhibitor, KD-IX-73-4, followed by incubation for 15 min at 37°C with or without 20 ng/ml TNF-α. ADAM-8 (■, *left axis*) and L-Selectin (▨, *right axis*) surface expression were measured by flow cytometry. Data shown represent the mean ± SD of the rMFI from five independent experiments. *, $p < 0.05$ by Wilcoxon signed rank test. *C*, Release of ADAM-8 by neutrophils. Cells were preincubated for 10 min at 37°C, in the absence (■) or presence (▨) of 20 μM KD-IX-73-4, followed by incubation with or without 20 ng/ml TNF-α or 20 ng/ml PMA for 15 or 30 min at 37°C. Soluble ADAM-8 was measured in the cell-free supernatant by ELISA. Data shown represent the mean ± SD of the relative ADAM-8 concentration from five independent experiments. *D*, Flow cytometry histogram of ADAM-8 surface expression in neutrophils adhered to endothelial cells. Confluent HUVEC were activated in the presence of 20 ng/ml TNF-α for 6 h and then, resting human neutrophils were allowed to attach to the endothelial cells under static conditions for 10 min at 37°C. Both neutrophils in suspension and attached to HUVEC were collected separately as described in *Materials and Methods*. The unshaded histograms represents the ADAM-8 expression of nonattached cells and the shaded histogram represents the ADAM-8 expression in attached neutrophils; the dotted histogram represents the negative control. A representative experiment of three is shown.

Results

Generation and characterization of anti-ADAM-8 mAbs

To address the regulation of ADAM-8 in neutrophils, we developed mAbs raised against a peptide containing the catalytic site of ADAM-8, showing <60% identity with its closest relative, ADAM-19. Among the Abs obtained, the anti-ADAM-8 mAb 2H5 was chosen for its reactivity against the Ag in immunoblots. The commercially available anti-hADAM-8 mAb was also tested in parallel. Western blot analysis using both mAbs revealed two major bands in resting human neutrophils, representing a proform of M_r = 120,000 and, a processed form with a M_r = 90,000, resulting from removal of the prodomain (Fig. 1*A*), in agreement with the previously described molecular weights for ADAM-8 (19). These Abs did not detect immunoreactive ADAM-8 in HUVEC lysates. To test the specificity of these two Abs, HEK293 cells were transiently transfected with a human ADAM-8 expression vector (pcDNA3-ADAM-8) (19) or with the empty parental vector (pcDNA3), as control. As shown by immunofluorescence (Fig. 1*B*), the anti-hADAM-8 mAb recognized Ag on the cell membrane of nonpermeabilized cells transfected with ADAM-8, but not of those transfected with the control plasmid. Likewise, J77 Jurkat cells were stably transfected with the same vectors and only the ADAM-8-transfected cells were specifically recognized by the anti-hADAM-8 mAb using flow cytometry (Fig. 1*C*). The 2H5 mAb did not recognize ADAM-8 by immunofluorescence or by flow cytometry on any of these ADAM-8-transfected cell lines as well as on resting or activated human neutrophils (data not shown).

As a first approach to determine the localization of ADAM-8 in human neutrophils, immunofluorescence studies showed that ADAM-8 was present at the plasma membrane of neutrophils (Fig. 1*D*). When these cells were permeabilized with 0.1% Triton X-100, ADAM-8 staining showed both membrane and vesicular pattern (Fig. 1*D*), suggesting that ADAM-8 could be stored in the intracellular granules of neutrophils.

Subcellular location of ADAM-8 in human neutrophils

To determine more precisely the subcellular location of ADAM-8, we performed subcellular fractionation assays under conditions that resolved cytosol, plasma membrane and the different granules of neutrophils. Anti-hADAM-8 mAb recognized in Western blot analysis a band of ~90 kDa in the postnuclear extract and in the membrane fraction of human neutrophils, but not in the soluble fraction containing the cytosol (Fig. 2*A*). This indicated that ADAM-8 was membrane bound. Fig. 2*B* shows distribution of protein markers in the different subcellular fractions from human resting neutrophils. ADAM-8 was broadly distributed, localized both at the plasma membrane and at the membranes prepared from the distinct cytoplasmic granules (Fig. 2*B*). ADAM-8 was mainly

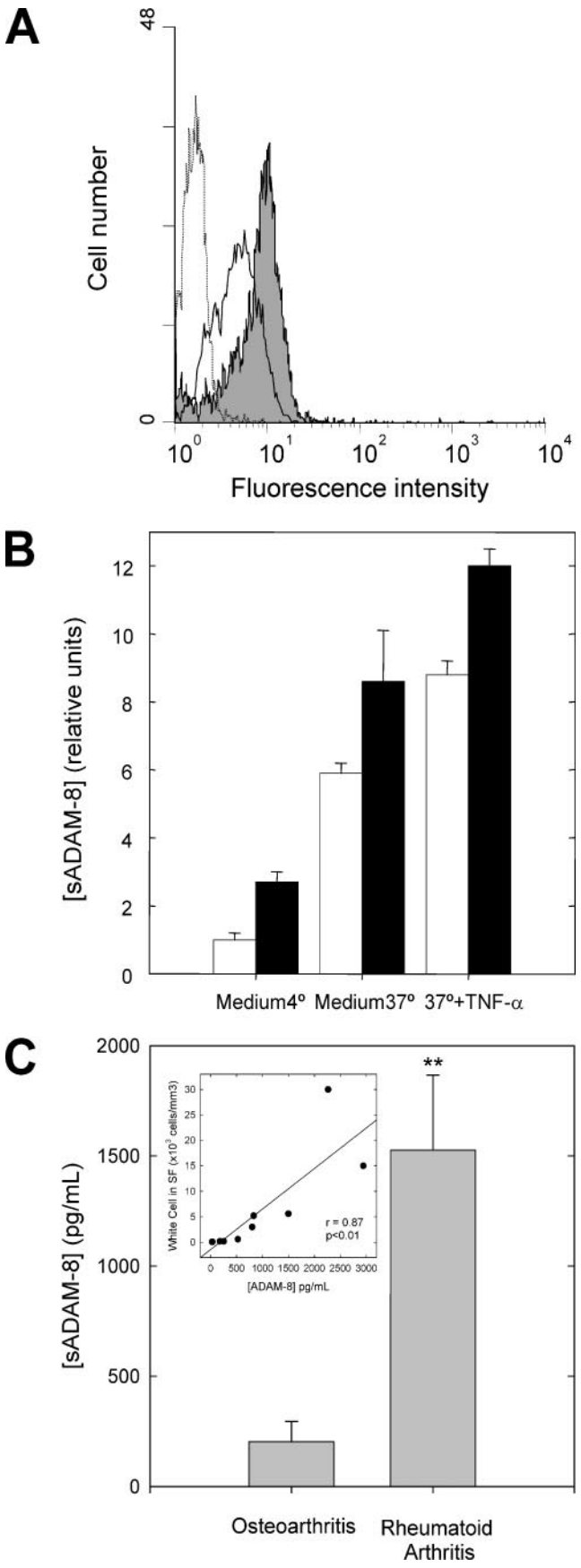

FIGURE 5. ADAM-8 expression is increased in synovial neutrophils and in synovial fluid obtained from RA patients. *A*, Flow cytometry analysis of ADAM-8 basal surface expression in neutrophils isolated from peripheral blood (unshaded histogram) and from synovial fluid of active RA patients (shaded histogram). Dotted histogram represents the negative control (P3X63 myeloma). A representative histogram of four is shown. *B*, ADAM-8 shedding by neutrophils from peripheral blood (□) and synovial fluid (■) of RA patients. Cells were isolated and maintained in medium at 4°C, as control, or incubated at 37°C. After 1 h, cell-free supernatants were collected and the concentration of sADAM-8 was determined by ELISA. TNF-α was used at 20 ng/ml. Results shown represent the mean ± SD content of sADAM-8, in relative units, of three independent experiments. *C*, Concentration of sADAM-8 in synovial fluid. Synovial fluid from knees of five RA and five osteoarthritis patients were assayed for sADAM-8 concentration by ELISA as described in *Materials and Methods*. Data represent the mean ± SD of the concentration of sADAM-8 in synovial fluid (picograms per milliliter). **, $p < 0.001$ vs osteoarthritis by Wilcoxon signed rank test. The plot shown in the *inset* represents the correlation between the increments of sADAM-8 and the total white cell count in synovial fluid from all patients studied. A highly significant direct correlation between the increment of sADAM-8 concentration and the cell count in synovial fluid ($r = 0.87$, $p < 0.01$) was found.

detected in subcellular fractions 4–6 (enriched in tertiary and specific granules), with secondary locations in fractions 2 and 3 (enriched in plasma membrane) and, to a lesser extent, in fractions 7 and 8 (enriched in azurophilic granules) (Fig. 2*B*). Because the subcellular fractionation conditions used could not resolve secretory vesicles from the plasma membrane (35), the localization of ADAM-8 in plasma membrane fractions could also include these subcellular organelles. When neutrophils were activated with PMA, which induces the selective secretion of specific and tertiary granules but not of azurophilic granules (36), a relative increase in the content of ADAM-8 in fraction 2 was observed (plasma membrane fraction), concomitant with a dramatic decrease in fractions 4–6 (tertiary and specific granules), which suggests that secretion of tertiary and specific granules during PMA stimulation incorporates ADAM-8-granule membrane bound to the cell surface. In fraction 7, and mainly in 8 (enriched in azurophilic granules), a relative increment in ADAM-8 immunoreactivity was observed (Fig. 2*B*). This apparent increase in the content of ADAM-8 in denser fractions might be explained by a putative fusion of intracellular granules during cell stimulation, a process that has been observed during neutrophils exocytosis (34, 37) as a part of the so-called compound exocytosis (38).

Immunogold electron microscopic analysis performed on resting neutrophils detected ADAM-8 at both the plasma membrane and in membranes from electron-dense granules and electron-lucent vesicles that include secretory vesicles (Fig. 3). ADAM-8 was detected on the outer side of the plasma membrane facing the external milieu (Fig. 3*A*), as well as in the inner side of the membrane of intracellular granules and vesicles (Fig. 3*B*). Using a direct counting of 16 randomized cell sections, the relative distribution of ADAM-8 in resting neutrophils was: 52% in electron-dense granules, 39% in electron-lucent vesicles, and 9% in plasma membrane. To confirm the location of ADAM-8 in the different neutrophil cytoplasmic granules and vesicles, cryosections of neutrophils were double labeled with anti-hADAM-8 mAb and specific markers for the different granules and vesicles. We found colocalization between ADAM-8 and albumin, a marker for secretory vesicles (Fig. 3*C*), ADAM-8 and gelatinase, a marker of tertiary granules (Fig. 3*D*), ADAM-8 and lactoferrin, a marker of specific granules (Fig. 3*E*), and ADAM-8 and myeloperoxidase, a marker for azurophilic granules (Fig. 3*F*). We also found that ADAM-8 localized to the phagolysosome of human neutrophils after phagocytosis of latex beads (Fig. 3*G*). Interestingly, the presence of doublets in the staining of ADAM-8 could be observed in several

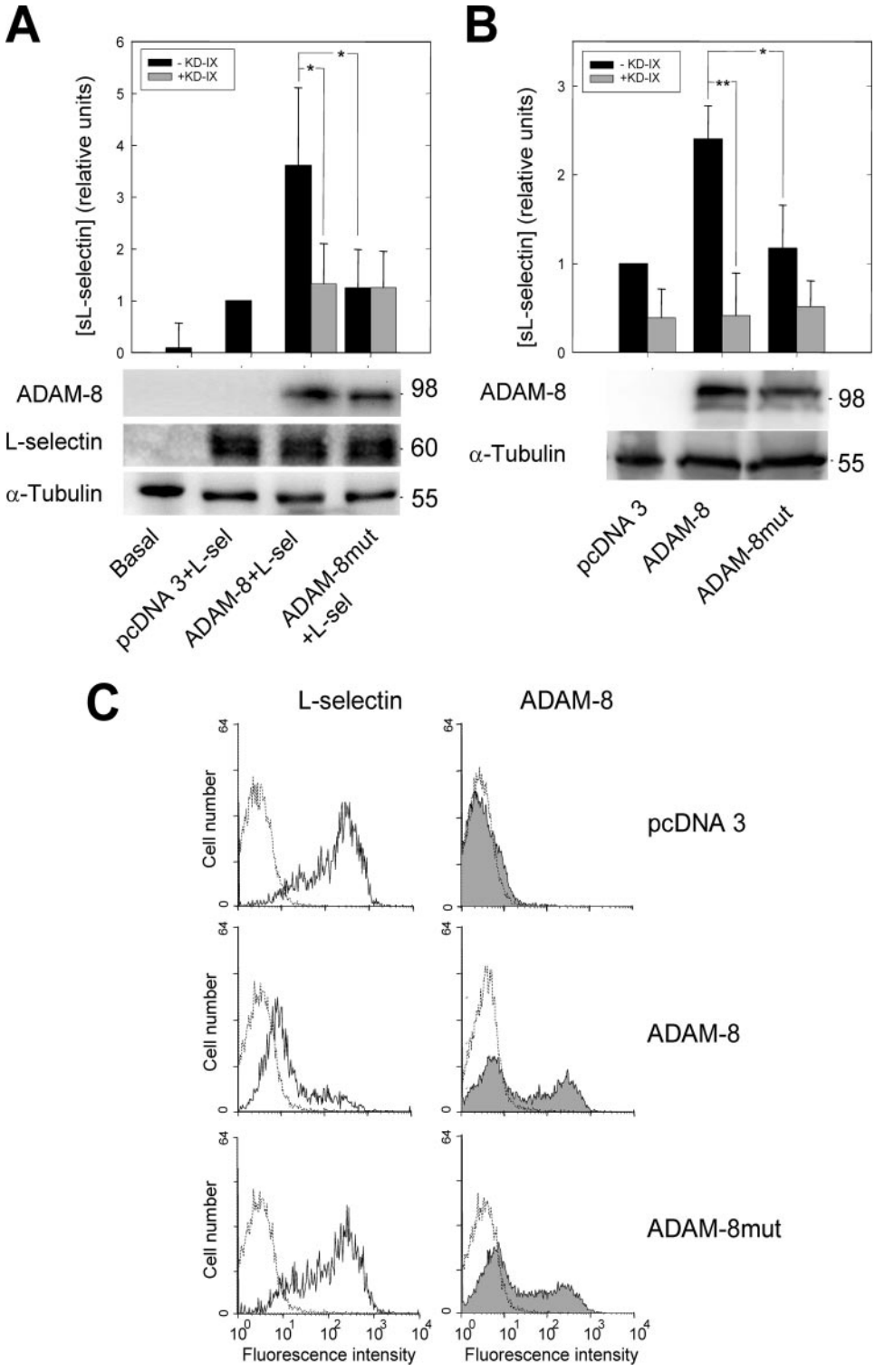

FIGURE 6. L-selectin shedding by membrane-bound ADAM-8. *A*, sL-selectin content in supernatants of HEK293 cells. Cells were cotransfected with an L-selectin expression vector with or without an expression vector encoding either a full-length (ADAM-8) or a catalytically inactive form of ADAM-8 (ADAM-8mut), as indicated. Cells were incubated for 48 h in absence (■) or presence (▨) of 20 μM KD-IX-73-4. The concentration of soluble ectodomain fragment of L-selectin was measured by ELISA in cell-free supernatant. Data represent the mean ± SE content of sL-selectin of six independent experiments in relative units. Western blots show the immunoreactivity of ADAM-8, L-selectin, and α-tubulin in cell lysates from one representative experiment. *B*, sL-selectin content in supernatants of CEM cells. Cells were transfected with or without an expression vector encoding either a full-length or a catalytically inactive form of ADAM-8 as indicated. Cells were incubated for 24 h in the absence (■) or presence (▨) of 20 μM KD-IX-73-4 and then, the quantity of soluble ectodomain fragments of L-selectin was assessed by ELISA. Data represent the mean ± SE of the relative sL-selectin concentration from four independent experiments. *, $p < 0.05$; **, $p < 0.001$ by Wilcoxon signed rank test. Western blots show the immunoreactivity of ADAM-8 and α-tubulin in cell lysates from one representative experiment. *C*, Flow cytometry analysis of L-selectin and ADAM-8 surface expression in CEM cells transfected with ADAM-8. Dotted histograms represent the negative control (isotype control); shaded histograms represent the surface expression of ADAM-8; unshaded histograms correspond to the L-selectin surface expression in cells after 24 h of transfection with empty vector (pcDNA 3), full-length or the catalytically inactive form of ADAM-8. One representative experiment of four is shown.

slides, suggesting high local molecule concentration (see *inset* in Fig. 3*D*, and Fig. 3, *A*, *B*, *E*, and *F*).

All these data demonstrate that ADAM-8 is located in both tertiary and specific granules as well as in secretory vesicles, all of which fuse with the plasma membrane following cell activation, and play a major role in the exocytic function of neutrophils (3, 4).

ADAM-8 is translocated to the cell membrane and secreted during neutrophil activation

The broad distribution of ADAM-8 in the different neutrophil granules and its enrichment at the plasma membrane after neutrophil activation strongly suggested that the translocation of this protein to the cell membrane was closely linked to the neutrophil degranulation process. To test this possibility, we analyzed the kinetics of cell surface expression of ADAM-8 by flow cytometry in neutrophils incubated at 37°C to trigger a progressive activation response. The basal cell surface expression of this metalloproteinase was transiently up-regulated in a time-dependent manner, reaching a peak after 15 min of incubation, and immediately followed by a significant reduction that reached a plateau at 45 min, showing a similar profile to that observed for the activation marker CD11b (39) (Fig. 4*A*). To clarify whether the decrease

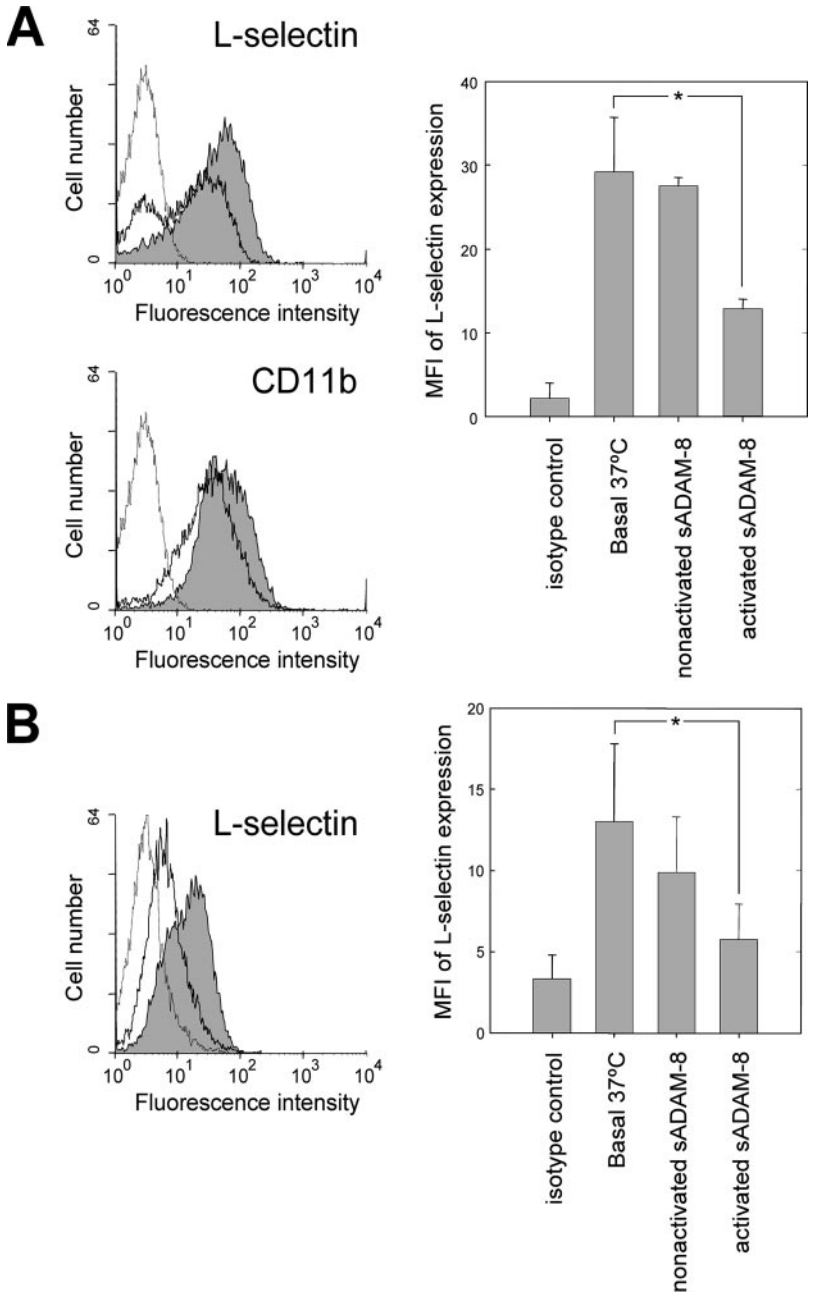

FIGURE 7. L-selectin expression is down-regulated by the soluble form of ADAM-8 in mammary cells. *A*, Flow cytometry analysis of L-selectin and CD11b surface expression in human neutrophils from peripheral blood. Cells were incubated in the presence of 40 μg/ml activated or nonactivated sADAM-8 for 30 min at 37°C, as described in *Materials and Methods*. The dotted histograms represent the negative control (isotype control), shaded histograms represent the L-selectin and CD11b expression in the presence of inactive sADAM-8. Unshaded histograms represent the L-selectin and CD11b expression of neutrophils incubated in the presence of active sADAM-8. A representative experiment of four is shown. Bars graph shows the MFI of L-selectin surface expression in neutrophils incubated with the active and inactive form of sADAM-8. Data represent the mean ± SD of four independent experiments. *B*, Flow cytometry analysis of L-selectin surface expression in an ADAM-17-deficient cell line. DRM ($^{-/-}$) cells were incubated for 3 h at 37°C in the presence of 40 μg/ml either active or inactive sADAM-8, as described in *Materials and Methods*. The dotted histogram represents the negative control (isotype control); the shaded histogram represents the basal L-selectin expression. The unshaded histogram represents the L-selectin expression of cells incubated in the presence of active sADAM-8. Bar graph shows the MFI of L-selectin surface expression in DRM$^{-/-}$ cells incubated with the active and inactive form of sADAM-8. Data represent the mean ± SD of four independent experiments. *, $p < 0.05$ by Wilcoxon signed rank test.

of ADAM-8 expression after the maximal peak was due to internalization, degradation, or release of the protein to the medium, we analyzed the expression of ADAM-8 on the cell surface after activation of the neutrophils in the presence or absence of the hydroxamic acid-based metalloproteinase inhibitor, KD-IX-73-4. As shown in Fig. 4*B*, the presence of KD-IX-73-4 prevented the temperature-induced loss of ADAM-8 cell surface expression. Similar results with the inhibitor were obtained when neutrophils were stimulated with TNF-α, indicating that the observed decrease of ADAM-8 expression at the plasma membrane after TNF-α activation was due to the shedding of the ADAM-8 ectodomain by a metalloproteinase. Under the same conditions, it was previously shown that KD-IX-73-4 also prevented the shedding of L-selectin (29). The loss of cell surface expression of ADAM-8 during neutrophil activation with TNF-α or PMA was accompanied by an increased release of ADAM-8 immunoreactivity in the culture supernatant in a time-dependent manner. This release was also prevented by pretreatment of cells with KD-IX-73-4 (Fig. 4*C*), further confirming a metalloproteinase-dependent release of ADAM-8 from the plasma membrane. In control experiments, ultracentrifugation of these supernatants fails to deplete sADAM-8 immunoreactive (data not shown), indicating that ADAM-8 was shed by a proteolytic mechanism rather than in membrane vesicles.

To analyze whether the surface expression of ADAM-8 might be modulated by intercellular interactions, an event that occurs early in the inflammatory response (1, 2), we studied the variation of cell surface expression of ADAM-8 in neutrophils attached to TNF-α-activated human endothelial cells isolated from umbilical cords (HUVEC). This assay showed that ADAM-8 membrane expression was up-regulated in neutrophils that were in close contact with endothelial cells, as compared with cells that had not established physical interaction with the activated endothelium (Fig. 4*D*). Similar results were obtained when neutrophils interacted with nonactivated HUVEC (data not shown).

ADAM-8 is up-regulated in neutrophils during the in vivo inflammatory response

Our results in this study show that in vitro activation of neutrophils by proinflammatory cytokines, phorbol esters, or by adhesion to HUVECs induced a rapid translocation of ADAM-8 from granules to the cell membrane, followed by its shedding by a metalloproteinase-dependent mechanism. To determine whether ADAM-8 could be regulated in a similar way during inflammatory response in vivo, we studied neutrophils simultaneously isolated from the synovial fluid and peripheral blood of patients with RA, a disease characterized by the chronic inflammation of joints. Neutrophils found in the synovial fluid expressed a higher amount of cell surface ADAM-8 than those isolated from peripheral blood (Fig. 5*A*). When these cells were further incubated at 37°C, the concentration of ADAM-8 detected by ELISA in the supernatant of neutrophils isolated from synovial fluid was higher than that from peripheral blood neutrophils (Fig. 5*B*). Neutrophils isolated from both compartments maintained the capability to up-regulate ADAM-8 shedding, as measured by an increase in the concentration of sADAM-8 upon TNF-α treatment (Fig. 5*B*). Analysis of the joint effusion from knees of five RA and five osteoarthritis patients showed that the synovial fluid from arthritis patients contained a concentration of sADAM-8 that was 6-fold higher than that observed from osteoarthritic knees (Fig. 5*C*). When the synovial fluid concentration of sADAM-8 was plotted vs the leukocyte count from the knee effusions from these patients, we found a highly significant direct correlation between the increment in sADAM-8 concentration and the cell count (correlation coefficient $r = 0.87$, $p < 0.01$) (*inset* Fig. 5*C*).

ADAM-8 mediates L-selectin shedding

After neutrophil activation, L-selectin is shed from the cell membrane, facilitating neutrophil extravasation from the bloodstream (6). Because ADAM-8 increase and L-selectin decrease were concurrent events on neutrophil cell membrane (Fig. 4*B*), we hypothesized that the ectodomain of L-selectin could be proteolytically cleaved, at least in part by ADAM-8 during neutrophil activation. To test this possibility, HEK293 cells were cotransfected with an expression vector encoding human wild-type L-selectin cDNA and with expression vectors bearing either the full-length (ADAM-8) or catalytically inactive (H604A, H608A) ADAM-8 (ADAM-8mut) cDNAs (19). An increase in the amount of sL-selectin was observed in the supernatant of cells cotransfected with L-selectin and ADAM-8 (Fig. 6*A*) when compared with cells cotransfected with L-selectin and ADAM-8mut or with cells transfected with L-selectin expression vector alone. The increment in sL-selectin concentration in the supernatant of cells expressing ADAM-8 was prevented by the metalloproteinase inhibitor KD-IX-73-4. Western blot analysis showed an equivalent amount of L-selectin in HEK293 cells transfected either with the wild-type or mutant ADAM-8 (Fig. 6*A*). When CEM cells, a lymphoblastic cell line that constitutively expresses L-selectin, were transfected with the ADAM-8 full-length, the amount of sL-selectin present in the cell-free supernatant was again increased respect to cells transfected with both the empty vector (pcDNA3) and the ADAM-8 catalytically inactive form. The presence of KD-IX-73-4 inhibitor prevented the increased shedding of L-selectin in ADAM-8 full-length transfected cells (Fig. 6*B*). The L-selectin surface expression level in CEM cells was clearly reduced when ADAM-8 full length, but not ADAM-8mut, were expressed at comparable levels on the cell surface (Fig. 6*C*).

The sADAM-8 has shown to be able to convert a number of membrane bound molecules into soluble forms (19, 20). To show cleavage of endogenous L-selectin by sADAM-8, human neutrophils were incubated for 20 min at 37°C in the presence of active or inactive form of sADAM-8. Active sADAM-8 caused a significant reduction in the cell surface expression of L-selectin, without up-regulation of CD11b in neutrophils (Fig. 7*A*).

The shedding of L-selectin is the result of its proteolytic cleavage conducted, at least in certain cell types and under certain conditions, by ADAM-17 (40). To ascertain the potential role of ADAM-17 in the down-regulatory activity of sADAM-8 on L-selectin, we assayed the effect of active sADAM-8 on $DRM^{-/-}$ cells, an ADAM-17-deficient mouse monocytic cell line that express human L-selectin. When this cell line was culture for 3 h at 37°C in the presence of active sADAM-8, an important reduction on the cell surface expression of L-selectin was observed. This effect on L-selectin surface expression was not observed in $DRM^{-/-}$ cell line with the inactive form of sADAM-8 (Fig. 7*B*).

Taken together, all these data suggest that both the membrane-bound and soluble forms of ADAM-8 are able to cleave the ectodomain of L-selectin. The release of L-selectin by ADAM-8 requires the integrity of its catalytic domain, the presence of proteolytically active form and it is independent of the presence of TACE.

Discussion

In this work, we describe the expression, localization, and regulation of ADAM-8 in neutrophils and its ability to shed L-selectin from the cell membrane. We show that: 1) ADAM8 is constitutively present in the membrane of all type of granules, in vesicles, and on cell surface of human neutrophils; 2) upon neutrophil activation, ADAM-8 is mobilized to the cell surface and rapidly shed to the extracellular milieu both in vitro and in vivo, by a metalloproteinase-dependent mechanism; 3) the expression of ADAM-8 on neutrophils is increased in inflammatory foci in vivo and levels of soluble ADAM-8 are proportional to the number of inflammatory cells; and 4) the presence of membrane-anchored and soluble active form of ADAM-8 is associated with an increase in the shedding of L-selectin in mammary cells. These findings suggest that ADAM-8 plays a key role in neutrophil function in innate immunity.

In human neutrophils, ADAM-8 is mainly present in tertiary and specific granules as well as in secretory vesicles, with secondary locations in both plasma membrane and azurophilic granules. This is the first description of a protein of the ADAM family in the secretory compartments of neutrophils and suggests that the metalloproteinase composition of these subcellular structures is more complex than expected, now including members of the ADAM family in addition to the previously described metalloproteinases such as matrix metalloproteinase (MMP)-8, MMP-9, and membrane type 6-MMP (41). This major presence of ADAM-8 in subcellular organelles that are readily mobilized to the plasma membrane, thus exposing this protein at the cell surface, could be a regulatory mechanism for the activity of ADAM-8 in neutrophils. The currently described substrates for ADAM-8 are located on the plasma membrane (19, 20) and thereby they can be only efficiently cleaved when degranulation of tertiary and specific granules, in response to proinflammatory stimuli, provides a sufficient amount of ADAM-8 on the plasma membrane. ADAM-8 was present in azurophilic granules, containing a large amount of proteolytic and microbicidal enzymes, that are sluggishly mobilized after cell activation and that are mainly directed to fuse with the phagosome during phagocytosis (3, 4). In this regard, we also found ADAM-8 in phagolysosomes of human neutrophils after phagocytosis of latex beads. The potential role that this metalloproteinase might play

in the process of phagocytosis and killing of microorganisms by neutrophils remains to be investigated.

The observed decrease of ADAM-8 on the cell membrane late after cell activation, and the presence of the enzyme in the cell supernatant, suggests secretion of the enzyme, rendering its soluble active form (18, 42). The release of ADAM-8, but not its mobilization to the cell membrane, was inhibited by the metalloproteinase inhibitor KD-IX-73-4, indicating that ADAM-8 was first mobilized to the cell membrane, and then processed by proteolysis and released to the milieu, in contrast with other granule metalloproteinases that are readily secreted during neutrophil activation (41). Although the metalloproteinase responsible for ADAM-8 processing is unknown, an autocatalytic capability has been described for this enzyme (13), suggesting that ADAM-8 could mediate its own secretion. The observation of ADAM-8 doublets in this work could fit with this view.

Neutrophils participate in innate immunity and tissue damage by releasing several catalytic enzymes and antimicrobial polypeptides from their intracellular granules (3). The activity of ADAM family members as major ectodomain sheddases of cell surface proteins, its implication in the cleavage of extracellular matrix components (11), in addition to functional activity described for sADAM-8 (18, 42) suggest that this metalloproteinase might play a role in the pathologic inflammatory response. ADAM-8 cell surface expression was up-regulated by the physical interaction of neutrophils with endothelial cells. This fact could suggest a regulatory role for this metalloproteinase in the initial phase of the adhesion cascade through the shedding of surface molecules that participate in the early neutrophil-endothelium interaction during the inflammatory response. In this context, neutrophils isolated from the synovial fluid of patients with active RA expressed and released a higher amount of ADAM-8 than neutrophils from peripheral blood. In addition, sADAM-8 concentration found in synovial fluid from patients with joint effusions was proportional to the degree of joint inflammation, which suggests that both the mobilization of ADAM-8 to the cell surface and its secretion might be induced by the migration and recruitment of neutrophils into the inflammatory foci. Because it has been described that other granule metalloproteinases play a role in extracellular matrix degradation (43), it is conceivable that the high expression of ADAM-8 in inflamed joints of patients with RA might contribute to the progress of joint degradation. This idea is supported both by the role that sADAM-8 has shown in the formation of OCL (18), cells involved in bone resorption, and by the fact that ADAM-8 is overexpressed in tissues around aseptically loosened bone implants (44). Thus, neutrophil expression and release of ADAM-8 might play a role in the development of tissue damage in chronic inflammatory diseases like RA, either by facilitating transendothelial neutrophil migration during the inflammatory response, and/or via a direct effect on extracellular matrix degradation of joint tissue.

L-selectin mediates rolling of leukocytes over the activated endothelium before firm adhesion. Although shedding of the L-selectin ectodomain is not essential for leukocyte recruitment, it does play a role in transendothelial extravasation (7). We have previously described that nonsteroidal anti-inflammatory drugs can induce the shedding of L-selectin, at least in part through the ADAM-17 (29). This member of the ADAM family cleaves a peptide corresponding to TNF-α, its best-characterized substrate, 2250-fold more efficiently than it cleaves peptides corresponding to L-selectin (40). This, and the fact that ADAM-17-deficient cells are able to release L-selectin from cell surface (9), suggest that ADAM-17 is not the main physiologic regulator of the rapid L-selectin shedding that happens in activated neutrophils (45), and that a metalloprotease activity other than ADAM-17 can mediate the cleavage of L-selectin. In this work, we show that the presence of ADAM-8 in the cell surface is associated with L-selectin shedding. This process was dependent on the catalytic activity of the enzyme, rather than the result of signaling cascades triggered through its disintegrin domain, because a mutation in the catalytic domain or the action of the metalloproteinase inhibitor KD-IX-73-4 abolished L-selectin release. In addition, the soluble form of ADAM-8 is also capable to induce the shedding of L-selectin in neutrophils without causing any significant effect on CD11b basal expression on neutrophils, indicating that this effect is not due to nonspecific cell activation (39). ADAM-17 and ADAM-8 have different cellular distribution and their expressions are regulated differently by neutrophil activation stimuli. ADAM-17 is constitutively expressed on the cell membrane of neutrophils, whereas ADAM-8 is localized both on the cell membrane and on the secretory granules. PMA induces a decrease in cell surface ADAM-17 by internalization, without producing the soluble form (46). On the contrary, PMA induces translocation of ADAM-8 to the cell membrane from the neutrophil granules and subsequent release of an active form of the enzyme. ADAM-17 and ADAM-8 have distinct substrate targets. TNF-α, the main substrate of ADAM-17, is not cleaved by ADAM-8, which is also unable to cleave other proinflammatory molecules like IL-1β or IL-6 (18). In contrast, CD23 and CHL1 are processed by ADAM-8 but not by ADAM-17 (19, 20). These results suggest that the shedding of L-selectin by ADAM-8 and ADAM-17 may take place in different pathological and physiological processes and may be regulated by different signals. In this regard, our results with an ADAM-17-deficient cell line indicate that the L-selectin shedding induced by ADAM-8, at least in its soluble form, is independent of ADAM-17 activity. We propose that, after recruitment, neutrophils are activated in contact with the endothelium and ADAM-8 is mobilized to the cell membrane, where it participates in the cleavage of L-selectin, facilitating neutrophil extravasation. In addition, it has been shown that soluble L-selectin decreases neutrophil migration (47, 48). Thus, ADAM-8-mediated L-selectin shedding could modulate the recruitment of additional leukocytes, acting as a negative feedback regulator.

In summary, we provide the first evidence of the localization, regulation, and function of ADAM-8 in neutrophils. Our results demonstrate that ADAM-8 forms part of the neutrophil proteolytic arsenal and that this metalloproteinase is involved in the shedding of L-selectin. Therefore, ADAM-8 might represent a target in the development of new anti-inflammatory therapies.

Acknowledgments

We thank Dr. A. G. Arroyo for experimental guidance. We are grateful to Dr. N. Borregaard, Dr. T. K. Kishimoto for providing key reagents, and to Dr. J. J. Peschon for the DRM$^{-/-}$ cell line. We are indebted to Dr. Manuel Feria for the critical reading of the manuscript and his continuous support.

Disclosures

The authors have no financial conflict of interest.

References

1. Butcher, E. C. 1991. Leukocyte-endothelial cell recognition: Three (or more) steps to specificity and diversity. *Cell* 67: 1033–1036.
2. Springer, T. A. 1994. Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. *Cell* 76: 301–314.
3. Borregaard, N., and J. B. Cowland. 1997. Granules of the human neutrophilic polymorphonuclear leukocyte. *Blood* 89: 3503–3521.
4. Mollinedo, F. 2003. Human neutrophil granules and exocytosis molecular control. *Inmunologia* 22: 340–358.
5. Burg, N., M. H. Pillinger, and S. B. Abramson. 2005. Neutrophils and eosinophils. In *Kelley's Textbook of Rheumatology.* E. D. Harris, R. C. Budd, M. K. Genovese, G. S. Firestein, J. S. Sargent, and C. B. Sledge, eds. Elsevier Saunders, Philadelphia, pp. 235–251.

6. Walcheck, B., J. Kahn, J. M. Fisher, B. B. Wang, R. S. Fisk, D. G. Payan, C. Feehan, R. Betageri, K. Darlak, A. F. Spatola, and T. K. Kishimoto. 1996. Neutrophil rolling altered by inhibition of L-selectin shedding in vitro. *Nature* 380: 720–723.
7. Smalley, D. M., and K. Ley. 2005. L-selectin: mechanisms and physiological significance of ectodomain cleavage. *J. Cell. Mol. Med.* 9: 255–266.
8. Peschon, J. J., J. L. Slack, P. Reddy, K. L. Stocking, S. W. Sunnarborg, D. C. Lee, W. E. Russell, B. J. Castner, R. S. Johnson, J. N. Fitzner, et al. 1998. An essential role for ectodomain shedding in mammalian development. *Science* 282: 1281–1284.
9. Walcheck, B., S. R. Alexander, C. A. St. Hill, and E. Matala. 2003. ADAM-17-independent shedding of L-selectin. *J. Leukocyte Biol.* 74: 389–394.
10. Yamamoto, S., Y. Higuchi, K. Yoshiyama, E. Shimizu, M. Kataoka, N. Hijiya, and K. Matsuura. 1999. ADAM family proteins in the immune system. *Immunol. Today* 20: 278–284.
11. White, J. M. 2003. ADAMs: modulators of cell-cell and cell-matrix interactions. *Curr. Opin. Cell Biol.* 15: 598–606.
12. Van Eerdewegh, P., R. D. Little, J. Dupuis, R. G. Del Mastro, K. Falls, J. Simon, D. Torrey, S. Pandit, J. McKenny, K. Braunschweiger, et al. 2002. Association of the *ADAM33* gene with asthma and bronchial hyperresponsiveness. *Nature* 418: 426–430.
13. Schlomann, U., D. Wildeboer, A. Webster, O. Antropova, D. Zeuschner, C. G. Knight, A. J. Docherty, M. Lambert, L. Skelton, H. Jockusch, and J. W. Bartsch. 2002. The metalloprotease disintegrin ADAM8. Processing by autocatalysis is required for proteolytic activity and cell adhesion. *J. Biol. Chem.* 277: 48210–48219.
14. Yoshiyama, K., Y. Higuchi, M. Kataoka, K. Matsuura, and S. Yamamoto. 1997. CD156 (human ADAM8): expression, primary amino acid sequence, and gene location. *Genomics* 41: 56–62.
15. Kelly, K., G. Hutchinson, D. Nebenius-Oosthuizen, A. J. Smith, J. W. Bartsch, K. Horiuchi, A. Rittger, K. Manova, A. J. Docherty, and C. P. Blobel. 2005. Metalloprotease-disintegrin ADAM8: expression analysis and targeted deletion in mice. *Dev. Dyn.* 232: 221–231.
16. Schlomann, U., S. Rathke-Hartlieb, S. Yamamoto, H. Jockusch, and J. W. Bartsch. 2000. Tumor necrosis factor α induces a metalloprotease-disintegrin, ADAM8 (CD156): implications for neuron-glia interactions during neurodegeneration. *J. Neurosci.* 20: 7964–7971.
17. King, N. E., N. Zimmermann, S. M. Pope, P. C. Fulkerson, N. M. Nikolaidis, A. Mishra, D. P. Witte, and M. E. Rothenberg. 2004. Expression and regulation of a disintegrin and metalloproteinase (ADAM) 8 in experimental asthma. *Am. J. Respir. Cell Mol. Biol.* 31: 257–265.
18. Choi, S. J., J. H. Han, and G. D. Roodman. 2001. ADAM8: a novel osteoclast stimulating factor. *J. Bone Miner. Res.* 16: 814–822.
19. Fourie, A. M., F. Coles, V. Moreno, and L. Karlsson. 2003. Catalytic activity of ADAM8, ADAM15 and MDC-L (ADAM28) on synthetic peptide substrates and in ectodomain cleavage of CD23. *J. Biol. Chem.* 278: 30469–30477.
20. Naus, S., M. Richter, D. Wildeboer, M. Moss, M. Schachner, and J. W. Bartsch. 2004. Ectodomain shedding of the neural recognition molecule CHL1 by the metalloprotease-disintegrin ADAM8 promotes neurite outgrowth and suppresses neuronal cell death. *J. Biol. Chem.* 279: 16083–16090.
21. Higuchi, Y., A. Yasui, K. Matsura, and S. Yamamoto. 2002. CD156 transgenic mice: different responses between inflammatory types. *Pathobiology* 70: 47–54.
22. Arnett, F. C., S. M. Edworthy, D. A. Bloch, D. J. McShane, J. F. Fries, N. S. Cooper, L. A. Healey, S. R. Kaplan, M. H. Liang, H. S. Luthra, et al. 1988. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. *Arthritis Rheum.* 31:3 15–324.
23. Galvez, B. G., S. Matias-Roman, J. P. Albar, F. Sanchez-Madrid, and A. G. Arroyo. 2001. Membrane type 1-matrix metalloproteinase is activated during migration of human endothelial cells and modulates endothelial motility and matrix remodeling. *J. Biol. Chem.* 276: 37491–37500.
24. Gomez-Gaviro, M. V., C. Dominguez-Jimenez, J. M. Carretero, P. Sabando, I. Gonzalez-Alvaro, F. Sanchez-Madrid, and F. Diaz-Gonzalez. 2000. Down-regulation of L-selectin expression in neutrophils by nonsteroidal anti-inflammatory drugs: role of intracellular ATP concentration. *Blood* 96: 3592–3600.
25. Feehan, C., K. Darlak, J. Kahn, B. Walcheck, A. F. Spatola, and T. K. Kishimoto. 1996. Shedding of the lymphocyte L-selectin adhesion molecule is inhibited by a hydroxamic acid-based protease inhibitor: identification with an L-selectin-alkaline phosphatase reporter. *J. Biol. Chem.* 271: 7019–7024.
26. Humbria, A., F. Diaz-Gonzalez, M. R. Campanero, A. G. Arroyo, A. Laffon, R. Gonzalez-Amaro, and F. Sanchez-Madrid. 1994. Expression of L-selectin, CD43, and CD44 in synovial fluid neutrophils from patients with inflammatory joint diseases: evidence for a soluble form of L-selectin in synovial fluid. *Arthritis Rheum.* 37: 342–348.
27. Rovida, E., A. Paccagnini, M. Del Rosso, J. Peschon, and P. Dello Sbarba. 2001. TNF-α-converting enzyme cleaves the macrophage colony-stimulating factor receptor in macrophages undergoing activation. *J. Immunol.* 166: 1583–1589.
28. Kinsella, T. M., and G. P. Nolan. 1996. Episomal vectors rapidly and stably produce high-titer recombinant retrovirus. *Hum. Gene Ther.* 7: 1405–1413.
29. Gomez-Gaviro, M. V., I. Gonzalez-Alvaro, C. Dominguez-Jimenez, J. Peschon, R. A. Black, F. Sanchez-Madrid, and F. Diaz-Gonzalez. 2002. Structure-function relationship and role of tumor necrosis factor-α-converting enzyme in the down-regulation of L-selectin by non-steroidal anti-inflammatory drugs. *J. Biol. Chem.* 277: 38212–38221.
30. Calafat, J., H. Janssen, M. Stahle-Backdahl, A. E. Zuurbier, E. F. Knol, and E. Egesten. 1997. Human monocytes and neutrophils store transforming growth factor-α in a subpopulation of cytoplasmic granules. *Blood* 90: 1255–1266.
31. Liou, W., H. J. Geuze, and J. W. Slot. 1996. Improving structural integrity of cryosections for immunogold labeling. *Histochem. Cell Biol.* 106: 41–58.
32. Slot, J. W., H. J. Geuze, S. Gigengack, G. E. Lienhard, and D. E. James. 1991. Immuno-localization of the insulin regulatable glucose transporter in brown adipose tissue of the rat. *J. Cell Biol.* 113: 123–129.
33. Kjeldsen, L., O. W. Bjerrum, D. Hovgaard, A. H. Johnsen, M. Sehested, and N. Borregaard. 1992. Human neutrophil gelatinase: a marker for circulating blood neutrophils: purification and quantitation by enzyme linked immunosorbent assay. *Eur. J. Haematol.* 49: 180–191.
34. Mollinedo, F., B. Martín-Martín, J. Calafat, S. M. Nabokina, and P. A. Lazo. 2003. Role of vesicle-associated membrane protein-2, through Q-soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor/R-soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor interaction, in the exocytosis of specific and tertiary granules of human neutrophils. *J. Immunol.* 170: 1034–1042.
35. Mollinedo, F., C. Gajate, and D. L. Schneider. 1991. Cytochrome *b* co-fractionates with gelatinase-containing granules in human neutrophils. *Mol. Cell. Biochem.* 105: 49–60.
36. Mollinedo, F., R. Pulido, P. M. Lacal, and F. Sanchez-Madrid. 1991. Mobilization of gelatinase-rich granules as a regulatory mechanism of early functional responses in human neutrophils. *Scand. J. Immunol.* 34: 33–43.
37. Lollike, K., M. Lindau, J. Calafat, and N. Borregaard. 2002. Compound exocytosis of granules in human neutrophils. *J. Leukocyte Biol.* 71: 973–980.
38. Scepek, S., and M. Lindau. 1993. Focal exocytosis by eosinophils—compound exocytosis and cumulative fusion. *EMBO J.* 12: 1811–1817.
39. Lacal, P., R. Pulido, F. Sanchez-Madrid, and F. Mollinedo. 1988. Intracellular location of T200 and Mo1 glycoproteins in human neutrophils. *J. Biol. Chem.* 263: 9946–9951.
40. Peschon, J. J., J. L. Slack, P. Reddy, K. L. Stocking, S. W. Sunnarborg, D. C. Lee, W. E. Russel, B. J. Castner, R. S. Johnson, J. N. Fitzner, et al. 1998. An essential role for ectodomain shedding in mammalian development. *Science* 282: 1281–1284.
41. Kang, T., J. Yi, A. Guo, X. Wang, C. M. Overall, W. Jiang, R. Elde, N. Borregaard, and D. Pei. 2001. Subcellular distribution and cytokine- and chemokine-regulated secretion of leukolysin/MT6-MMP/MMP-25 in neutrophils. *J. Biol. Chem.* 276: 21960–21968.
42. Amour, A., C. Knight, W. English, A. Webster, P. Slocombe, V. Knauper, A. Docherty, J. Becherer, C. Blobel, and G. Murphy. 2002. The enzymatic activity of ADAM8 and ADAM9 is not regulated by TIMPs. *FEBS Lett.* 524: 154–158.
43. Yamanishi, Y., and G. S. Firestein. 2001. Pathogenesis of rheumatoid arthritis: the role of synoviocytes. *Rheum. Dis. Clin. North Am.* 27: 355–371.
44. Mandelin, J., T. F. Li, M. V. Hukkanen, M. Liljestrom, Z. K. Chen, S. Santavirta, U. Kitti, and Y. T. Kontinen. 2003. Increased expression of a novel osteoclast-stimulating factor, ADAM8, in interface tissue around loosened hip prostheses. *J. Rheumatol.* 30: 2033–2038.
45. Kishimoto, T. K., M. A. Jutila, E. L. Berg, and E. C. Butcher. 1989. Neutrophil Mac-1 and MEL-14 adhesion proteins inversely regulated by chemotactic factors. *Science* 245: 1238–1241.
46. Doedens, J. R., and R. A. Black. 2000. Stimulation-induced down-regulation of tumor necrosis factor-α converting enzyme. *J. Biol. Chem.* 275: 14598–14607.
47. Ferri, L. E., D. Swartz, and N. V. Christou. 2001. Soluble L-selectin at levels present in septic patients diminishes leukocyte-endothelial cell interactions in mice in vivo: a mechanism for decreased leukocyte delivery to remote sites in sepsis. *Crit. Care Med.* 29: 117–122.
48. Watson, S. R., C. Fennie, and L. A. Lasky. 1991. Neutrophil influx into an inflammatory site inhibited by a soluble homing receptor-IgG chimaera. *Nature* 349: 164–167.