Eur Respir J, 1993, 6, 791–796
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European Respiratory Journal
ISSN 0903 - 1936

Leucocyte elastase-mediated release of von Willebrand factor from cultured endothelial cells

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Leucocyte elastase-mediated release of von Willebrand factor from cultured endothelial cells. M. Chignard, V. Balloy, P. Renesto. ©ERS Journals Ltd 1993.

ABSTRACT: The aim of this study was to investigate a possible activation of human endothelial cells in monolayer culture by a purified neutrophil-derived proteinase, *i.e.* elastase.

Cells were isolated from human umbilical cord veins, and incubated either in primary culture or after two passages, in the presence of various concentrations of this proteinase.

Although a lack of prostacyclin formation was noted, elastase induced a large release of von Willebrand factor (vWf) in a concentration-dependent manner. Thus, upon incubation with 1 μg·ml⁻¹ elastase for 30 min, 70 mU·ml⁻¹ vWf were detected in the incubation medium, as compared to 5 mU·ml⁻¹ for control. Using cells in primary culture, a fivefold higher concentration of vWf was recovered following incubation with 10 μg·ml⁻¹ elastase than with 0.5 IU·ml⁻¹ thrombin. This effect was linked to the enzymatic activity of elastase and not to its cationic charge, as deduced from the inhibition by eglin C, and the lack of effect of the phenylmethylsulphonyl fluoride (PMSF) treated proteinase.

We concluded that vWf release was not due to cell activation, since cytoplasmic calcium mobilization was absent, and inhibition of protein kinase C did not modify the response. In fact, this release was the consequence of cell damage, since concentrations of vWf recovered correlated with cell lysis.

These results support the hypothesis that the high level of plasma vWf in patients with sepsis or adult respiratory distress syndrome could result from damage to the endothelial cells by elastase released from activated neutrophils.

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Keywords: Human endothelial cells
leucocyte elastase,
von Willebrand factor

Received: July 1 1992
Accepted after revision January 26 1993

Polymorphonuclear neutrophils (PMN) contain in their azurophilic granules several enzymes, among which is elastase (EC 3. 4. 21. 37.), a cationic serine proteinase [1]. This proteinase, which is released upon cell activation, may play a biological role in the direct environment of circulating PMN. For example, elastase participates in the PMN-mediated activation of nearby platelets under *in vitro* experimental conditions (Renesto and Chignard, submitted for publication). Since other proteinases, such as thrombin or trypsin which activate platelets also activate endothelial cells in terms of prostacyclin (PGI_2) formation [2], and von Willebrand factor (vWf) release [3], we sought for such an activation by elastase, using human umbilical vein endothelial cells (HUVEC) in culture. Such a demonstration could be relevant to the *in vivo* situation, particularly in a pathological state, such as septicemia, sepsis syndrome, or the adult respiratory distress syndrome. For example, this latter disease, which appears to be related to host injury phenomena secondary to PMN activation, is characterized, among other features, by an increase of elastase, PGI_2 stable end product [4], and vWf [5], in the plasma.

Materials and methods

Materials

Human umbilical cords were obtained from the maternity departments of Hôpital Rothschild and Hôpital Boussicaut, Paris, France. Recombinant eglin C was obtained from Ciba-Geigy (Basel, Switzerland) through the courtesy of H.P. Schnebli. α_1-antitrypsin was a gift from J.G. Bieth from Inserm no. 237 (Strasbourg, France). Aprotinin, phenylmethylsulphonyl fluoride (PMSF), heparin, N-succinyl-ala-ala-ala-p-nitroanilide, staurosporine and histamine dihydrochloride were purchased from Sigma Chemical Corp. (St. Louis, MO, USA); heat-inactivated fetal calf serum (FCS) and L-glutamine from Jacques Boy, (Reims, France); medium 199 containing Earle's salt (M199), $NaHCO_3$ and hydroxyethylpiperazine ethanmesuplhonic acid (Hepes) from Eurobio (Les Ulis, France); Hanks' balanced salt solution (HBSS), penicillin, streptomycin and amphotericin B from Gibco/BRC SARL (Cergy Pontoise, France); human serum albumin (HSA)

from Biotransfusion (Roissy, France); CM-Trisacryl M from IBF biotechnics (Villeneuve la Garenne, France); Fura 2-acetoxymethylester from Calbiochem Corp. (San Diego, USA) and thrombin from Roche (Neuilly/Seine, France). The kit for vWf determination was from Diagnostica Stago (Asnières, France). Collagenase H and endothelial cell growth factor (ECGF) were from Boehringer Mannheim (Mannheim, Germany). Twenty four and 96-well Falcon primaria plastic tissue culture plates and plastic tissue culture flasks (T25 and T75) were purchased from Becton Dickinson (New Jersey, USA). Antibodies anti-6-keto prostaglandin F1α ($PGF_{1\alpha}$) and the iodinated 6-keto $PGF_{1\alpha}$ were obtained from URIA, Institut Pasteur (Paris, France), and $Na^{51}CrO_4$ was from Amersham (Amersham, Buckinghamshire, UK).

Preparation of HUVEC monolayers

HUVEC were isolated from human umbilical veins according to the method originally described by JAFFE *et al.* [6]. Briefly, following collagenase digestion, cells were cultured in M199 containing 10 mM Hepes, 14 mM $NaHCO_3$ and supplemented with 20% FCS, 100 $U{\cdot}ml^{-1}$ penicillin, 100 $\mu g{\cdot}ml^{-1}$ streptomycin, 0.25 $\mu g{\cdot}ml^{-1}$ amphotericin B, 2 mM L-glutamine, 50 $\mu g{\cdot}ml^{-1}$ porcine heparin and 20 $\mu g{\cdot}ml^{-1}$ ECGF. Cells were grown to confluence in 96-well plates, when used as primary culture. When used after two passages, cells were successively seeded in T25 flasks and used at confluence in 24-well plates. Passages were performed following a brief exposure to 1.25% trypsin.

Purification of elastase

Elastase (human leucocyte elastase - HLE) was purified from PMN granules as described previously [7]. Briefly, a crude granule extract of PMN was prepared, and subsequently processed through an aprotinin-sepharose column. Elastase was desorbed from the affinity chromatography column by elution with 0.05 M sodium acetate, 1 M NaCl, pH 5.5. After concentration and dialysis, the mixture was applied onto a CM-Trisacryl M column, and the elution was carried out (buffer composition: 0.05 M sodium acetate, 0.45 M NaCl, pH 5.5) in order to elute elastase. The enzymatic activity of elastase was determined spectrophotometrically using N-succinyl-ala-ala-ala-p-nitroanilide as a specific substrate. For the determination of the active site concentration, a fixed volume of the enzyme was mixed with increasing amounts of active site titrated α_1-antitrypsin. After 1 min incubation at 37°C, residual enzymatic activities were measured. Proteinase active site concentrations were evaluated by extrapolation of the inhibition curve obtained. The purified batches of elastase migrated on polyacrylamide gel electrophoresis (PAGE) (Phast Gel Gradient 10–15, Phast System Separation, Pharmacia, Sweden) with an apparent molecular weight of 28 kD. For some experiments, elastase (2 $mg{\cdot}ml^{-1}$), was incubated for 60 min at room temperature with PMSF (1.25 mM), and then dialysed using a microconcentrator to remove the inhibitor. Following this treatment, elastase was devoid of enzymatic activity as assessed by measuring hydrolysis of its specific synthetic substrate.

Incubation of HUVEC with elastase

Studies were performed on confluent cultures, previously washed twice with M199 without FCS. Cells were then incubated with 100 µl of the same medium, containing elastase at the required concentrations. Following a 30 min incubation period at 37°C in a humidified atmosphere of air/CO_2 (19:1), cell free supernatants were collected and kept at -20°C until 6-keto $PGF_{1\alpha}$ or vWf measurements were performed.

Measurement of 6-keto $PGF_{1\alpha}$

Synthesis of PGI_2 was assessed by measuring the concentration of 6-keto $PGF_{1\alpha}$, its stable metabolite, in the incubation mediums by means of radioimmunoassay. Mediums were incubated at 4°C overnight, with anti-6-keto $PGF_{1\alpha}$ antibodies and a fixed concentration of iodinated 6-keto $PGF_{1\alpha}$. Separation of the free from the antibody-bound eicosanoid was carried out by addition of polyethyleneglycol 6000 (30% in distilled water), followed by centrifugation (1,450×g for 10 min at 4°C). Radioactivity values of the pellets, corresponding to the bound fractions, were measured with a γ counter (Kontron Analytical MDA 312), and were used to calculate 6-keto $PGF_{1\alpha}$ concentrations by projection on a standard curve, established with fixed concentrations of unlabelled material.

Measurement of vWf

The amounts of vWf released were determined from HUVEC incubation medium, using an enzyme immunoassay. Briefly, microwells were coated with specific rabbit anti-vWf antibodies, and samples were then added. Two hours later, washings were performed, and rabbit anti-vWf antibodies coupled to peroxidase were added to form a sandwich. Finally, the bound enzyme peroxidase was revealed by its activity on its substrate, orthophenylenediamine, in the presence of hydrogen peroxide. Values were calculated from a standard curve, established with a vWf-IX:Ag control pooled plasma, provided by the manufacturer. One unit of vWf was defined as the amount present in 1 ml of the pooled plasma, diluted 1:50 as recommended.

Measurements of cell detachment and lysis

For these studies, cells were preincubated for 18 h with $Na^{51}CrO_4$ (10 µM) in M199. Just before performing experiments, cells were washed, as described above, for incubation with elastase. At the end of the 30 min incubation period, mediums were collected and centrifuged

(300×g, 10 min), in order to evaluate cell lysis (supernatant), and detachment (pellet). Values were expressed as percentage of the total radioactivity per well, obtained by the addition of Triton X-100 (1% final concentration) to the plated monolayer and counting the whole suspension.

Measurements of intracytoplasmic calcium movements

HUVEC in primary culture were detached by incubation with 1.5 mM ethylenediamine tetra-acetic acid (EDTA) in HBSS at 37°C for 30 min. Cells were then scraped with a rubber-policeman and the medium was collected, mixed with 4 volumes HBSS and centrifuged (300×g, 10 min). The pellet was resuspended in HBSS at a final concentration of 2×10^6 cells·ml^{-1} and incubated with Fura 2-acetoxymethylester (5 μM for 30 min at 37°C). After two washings, cells were resuspended in a volume of HBSS so that the final concentration was of 2×10^6 cells·ml^{-1}. Fura 2-loaded cells in suspension (0.5 ml) were then mixed with an equal volume of HBSS, supplemented with $CaCl_2$ (2.6 mM) and $MgCl_2$ (2 mM), into a quartz spectroscopy cuvette inserted into a spectrofluorimeter (Jobin Yvon JY 3D), thermostated at 37°C under stirring. Fluorescence excitation and emission wavelengths were 340 nm (4 nm slits) and 510 nm (10 nm slits), respectively. Elastase (5 or 10 μg·ml^{-1}) or thrombin (1 IU·ml^{-1}) were added, and fluorescence tracings were recorded. At the end of each stimulation, the calibration for Fura 2 signal was performed by lysing cells with saponin (1 mg·ml^{-1}) to obtain the maximal fluorescence value (F_{max}), followed by the quenching of Fura 2 associated fluorescence with 30 mM Tris base and 5 mM ethylene glycol tetra-acetic acid (EGTA) to obtain the minimal fluorescence value (F_{min}). Intracellular free calcium concentrations were calculated from the equation of Grynkiewicz *et al.* [8], using a Kd of 224 nM.

Statistics

Results are expressed as mean±SD of at least three distinct experiments. Statistical analysis was performed by Student's t-test. Results are significant at a value of $p<0.05$*.

Results

Formation of prostacyclin

HUVEC obtained after two passages were incubated for 30 min with increasing concentrations of elastase, ranging 0.1–15 μg·ml^{-1}. They were unable to trigger PGI_2 formation. As shown in figure 1a, concentrations of the PGI_2 metabolite, 6-keto $PGF_{1\alpha}$ recovered in the incubation medium were not significantly different from those reached in control incubates, due to a spontaneous biosynthesis. By contrast, upon incubation with thrombin (0.5 IU·ml^{-1}), a fourfold quantity of 6-keto $PGF_{1\alpha}$ was recovered.

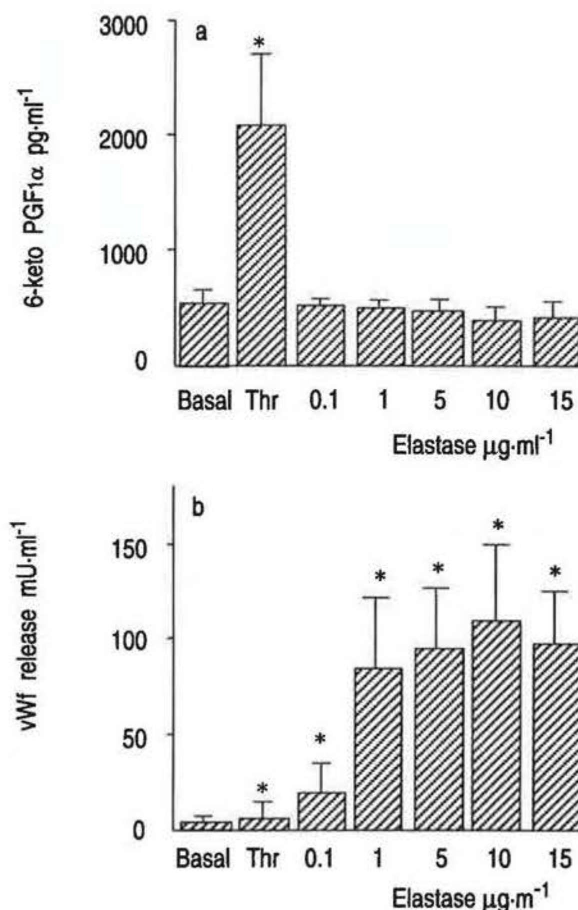

Fig. 1. – Effect of elastase on PGI_2 synthesis and vWf release by HUVEC. HUVEC at the second passage were incubated without (control) or with elastase (at different concentrations expressed in μg·ml^{-1}) for 30 min at 37°C. For each experiment, a positive control was run using thrombin (0.5 IU·ml^{-1}) as a standard agonist. a) The stable catabolite of PGI_2, 6-keto $PGF_{1\alpha}$; and b) vWf were quantitated from corresponding supernatants. Each histogram is the mean±SD of 5–6 distinct experiments. *: $p<0.05$. PGI_2: prostacyclin; vWf: von Willebrand factor; HUVEC: human umbilical vein endothelial cells; $PGF_{1\alpha}$: prostaglandin $F_{1\alpha}$; Thr: thrombin.

Release of von Willebrand factor

Under the same experimental conditions as those reported for 6-keto $PGF_{1\alpha}$ determination, vWf was found in significant amounts in mediums of elastase-treated HUVEC. As illustrated in figure 1b, the concentrations detected were dependent on the concentrations of elastase added. Since this result contrasted with the absence of PGI_2 formation, another study was undertaken using HUVEC in primary culture. As shown in figure 2, similar data were obtained, the smallest effective elastase concentration being 0.5 μg·ml^{-1} (16.7 nM). By comparison, under the same experimental conditions, both histamine (0.1 mM) and thrombin (0.5 IU·ml^{-1}), two well-known

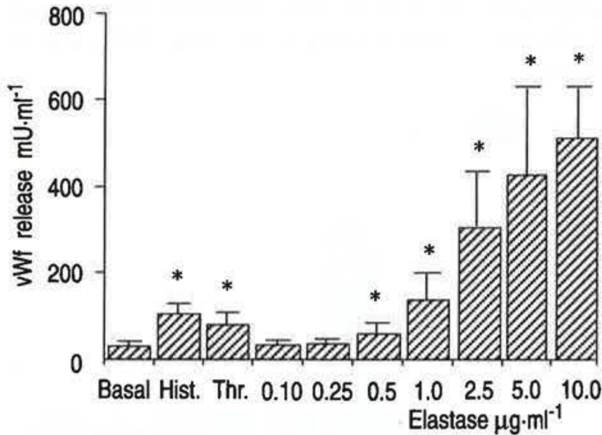

Fig. 2. – Effect of elastase on vWf release from HUVEC in primary culture. HUVEC in primary culture were incubated without (control), or with elastase (at different concentrations expressed in µg·ml⁻¹) for 30 min at 37°C. For each experiment, a positive control was run using histamine (0.1 mM) and thrombin (0.5 IU·ml⁻¹) as standard agonists. Each histogram is the mean±SD of 5–6 distinct experiments. *: $p<0.05$. Hist: histamine. For further abbreviations see legend to figure 1.

HUVEC agonists, also induced significant release but they were approximately a fifth of those observed with the highest concentration of elastase (10 µg·ml⁻¹; 332 nM).

Effect of proteinase inhibitors

Eglin C, a proteinase inhibitor extracted from leeches, is specifically effective against the enzymatic activity of elastase [9]. As shown in Fig. 3 when 10 µg·ml⁻¹ elastase were preincubated with recombinant eglin C (25 µg·ml⁻¹) for 5 min and then added to HUVEC for 30 min, vWf release was suppressed. In addition, when PMSF-treated elastase (10 µg·ml⁻¹) *i.e.* an active site blocked elastase, was used in place of the native proteinase, no release of vWf was observed (fig. 3).

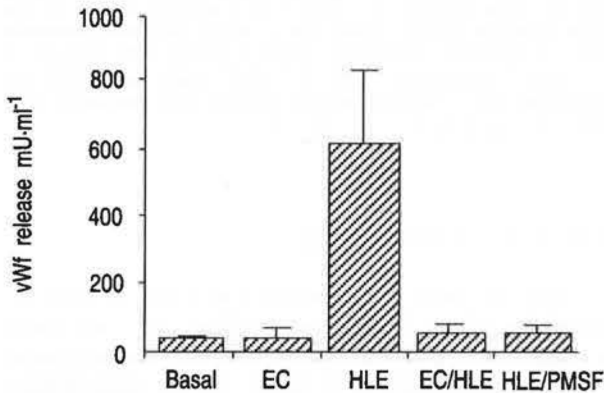

Fig. 3. – Effect of the inhibition of the enzymatic activity of elastase on vWf release. Experiments were performed as described in figure 1. HUVEC were treated for 30 min with elastase (10 µg·ml⁻¹) preincubated for 5 min with eglin C (25 µg·ml⁻¹) or treated with PMSF. For each experiment, a positive control was run using the native proteinase (10 µg·ml⁻¹). Results are means±SD of 5–6 distinct experiments. HLE: human leucocyte elastase; EC: eglin C; PMSF: phenylmethylsulphonyl fluoride. For further abbreviations see legend to figure 1.

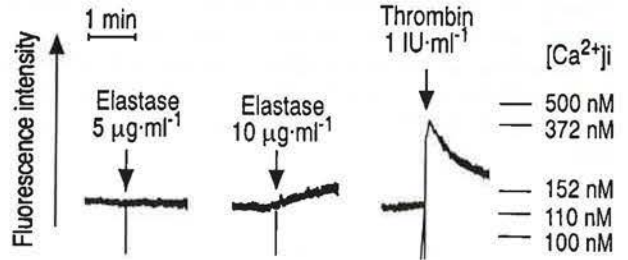

Fig. 4. – Effect of elastase on cytoplasmic calcium movements. HUVEC (from primary culture) in suspension (10^6 cells·ml⁻¹) were preloaded with Fura-2 and challenged with elastase or thrombin (1 IU·ml⁻¹). Tracings represent fluorescence intensity variations in function of time (min) and correspond to one experiment representative of three others. HUVEC: human umbilical vein endothelial cells.

Mechanism of action

Firstly, calcium mobilization within the cell in suspension was studied. Although a calcium flux was triggered by thrombin, it was impossible to observe one with elastase, under similar circumstances (fig. 4). Since vWf release can also occur *via* a direct stimulation of protein kinase C (PKC) [10], experiments were performed with HUVEC monolayers preincubated with staurosporine, a potent PKC inhibitor. Under optimal effective conditions, (*i.e.* 10 µM for 10 min [11]), staurosporine was unable to significantly modify the release of vWf induced by 5 µg·ml⁻¹ elastase: 429±60 *vs* 425±61 mU·ml⁻¹ for control experiments.

A cytotoxic effect for the proteinase was then investigated. Whatever the preparation of HUVEC used (after two passages or in primary culture), elastase between 2.5 µg·ml⁻¹ and 10 µg·ml⁻¹ induced significant concentration-dependent cell detachment and lysis, the maximum values being 16.6±9.3% and 22.1±6.5%, respectively. As shown in figure 5, there was a strong correlation ($r=0.98$, $p<0.001$) between the release of vWf and the lysis of HUVEC in primary culture.

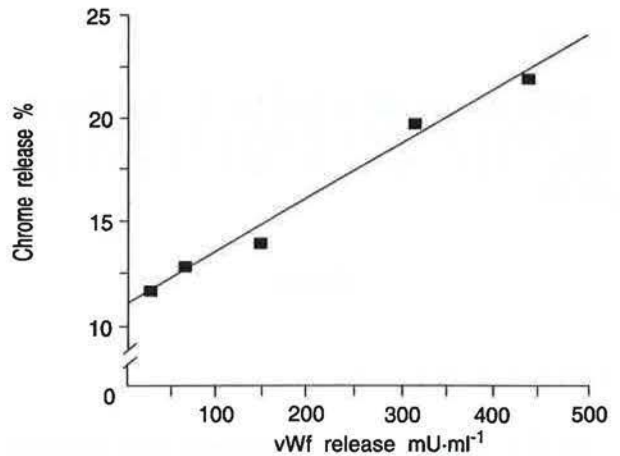

Fig. 5. – Correlation between vWf release and cell lysis induced by elastase. Experiments were performed as described in figure 1, using HUVEC in primary culture preloaded with $Na^{51}CrO_4$. Percentages of released radiolabelled chromium were plotted against concentrations of released vWf (values from figure 2 for each elastase concentration). Values are means of 5–6 distinct experiments ($r=0.98$; $p<0.001$). For abbreviations see legend to figure 1.

Discussion

Incubation of HUVEC in monolayer culture after two passages with elastase did not trigger PGI_2 formation within 30 min. These data were in agreement with two previous works published by LeRoy *et al.* [12] and Weksler *et al.* [13], who noted little or no effect. In contrast, we observed an accumulation of vWf in the cell incubation medium, this molecule being stored in the Weibel-Palade bodies of cells. It was previously reported that vWf was rapidly released in a calcium-dependent manner upon activation by biological agonists, such as thrombin or histamine [14]. It was, thus, paradoxical to observe such a release without PGI_2 formation, another well-known calcium-mediated pathway.

Using HUVEC in primary culture, we confirmed the presence of vWf in the medium following incubation with elastase. The recovered concentrations were even higher than those recovered after histamine or thrombin stimulation under the same experimental conditions. In order to delineate the mechanism by which elastase was inducing this release, the involvement of the enzymatic activity was checked. The use of recombinant eglin C which complexes elastase [9], indicated that enzymatic activity was probably involved, since elastase failed to induce vWf release when eglin C was present in the medium. Blockade of the enzymatic site by PMSF treatment, confirmed such a mechanism of activity and, at the same time, ruled out a potential effect of this strongly cationic protein [1] through charge-related interactions. The next step was to study the intracellular mechanism by which elastase triggered vWf release. Surprisingly, no calcium movements could be detected upon elastase challenge, although HUVEC were responsive to thrombin.

These data, as well as the lack of involvement of PKC, cast a doubt on a true activation of the cells by elastase, and led us to study a possible cytotoxic effect under our experimental conditions. Indeed, cell detachment and, more importantly, cell lysis were observed. In fact, the range of concentrations inducing lysis was superimposable to the range inducing vWf release, and a high correlation was found between both parameters, leading to the conclusion that elastase was inducing vWf release through a lytic process. In fact, it has previously been shown that activated PMN induced cell detachment [15, 16], and cell lysis [17, 18] *via* proteinases. Indeed, proteinase inhibitors were able to counteract these effects, and incubation of endothelial cells in culture with purified elastase resulted in cell detachment [19], and lysis [17, 18].

In previous experiments, we have shown that concentrations of elastase up to 340 nM (*i.e.* approximately 10 $\mu g \cdot ml^{-1}$) can be found in the environment of 5×10^6 activated $PMN \cdot ml^{-1}$ (Renesto and Chignard, submitted for publication). It can, thus, be assumed that the mechanism that we observed *in vitro* with HUVEC in culture could be operative *in vivo* although blood provides potent specific elastase inhibitors, particularly α_1-antitrypsin (or α_1-proteinase inhibitor). Nonetheless, it can be hypothesized that in capillaries, elastase is released at the interface between PMN and endothelial cells, thus giving an even higher local concentration, and preventing access for specific inhibitors by the formation of a microenvironment. This later mechanism is likely to occur, since it has been shown that proteinases released from PMN can react with susceptible substrates even in the presence of physiological concentrations of proteinase inhibitors [20, 21]. The most relevant demonstration has been given by Smedly *et al.* [17]. These authors clearly showed that endotoxin-pretreated PMN, once activated by specific stimuli, triggered endothelial cell detachment and lysis. This injury was inhibited by a specific elastase synthetic inhibitor, but not by serum or plasma. Furthermore, they also proved that endotoxin was priming PMN, and was not directly altering endothelial cell integrity or increasing its susceptibility to PMN-mediated killing.

The effect of elastase on HUVEC described here, could well occur under some pathological circumstances. Indeed, a significant granulocytic proteolytic activity has been detected in the plasma of patients with septicaemia [22] on the one hand, and an elevated level of plasma vWf is present in patients with sepsis syndrome, on the other [23]. In fact, in the latter report, authors indicated that this increase is an early predictor of acute lung injury, a pathology for which a high plasmatic concentration of vWf is a characteristic feature [5]. More interestingly, it has been demonstrated that the injection of endotoxin to normal subjects led to an increase in vWf plasmatic concentrations up to fourfold by three hours [24]. A possible explanation was that endotoxin was directly acting on endothelial cells under these *in vivo* conditions, as observed *in vitro* by Harlan *et al.* [25], although these results were not confirmed by Smedly *et al.* [17]. In fact, elastase could be the mediator responsible, since this proteinase was also released with a similar time course from PMN, as detected by an increase in circulating elastase-α_1-antitrypsin complexes [24]. Endotoxin administration to animals provoked activation of PMN, elastase release, and changes in capillary endothelium, among other features, leading to acute lung injury [26]. These experimental studies are currently used as a model for the adult respiratory distress syndrome, a pathology in which the presence of vWf has been noted in circulating blood [27]. Thus, it can be hypothesized: 1) that the presence of vWf in patients could result from an effect of elastase released from activated PMN on endothelial cells, and 2) that this effect could be the consequence of damage rather than an activation of endothelial cells.

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