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Protective effect of platelet activating factor antagonists on cultured endothelial cell lysis induced by elastase or activated neutrophils

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1 The mechanism(s) responsible for injury of endothelial cells induced by human leukocyte elastase (HLE) was investigated in an immortalized venous human endothelial cell line (IVEC).

2 First, the proteinase concentrations and incubation delays necessary to trigger a significant IVEC cytotoxicity were determined by chromium assays. Thus, exposure of IVEC for 6 h to 10 $\mu g\ ml^{-1}$ HLE resulted in $22 \pm 2.8\%$ lysis and $36.4 \pm 5.4\%$ detachment (mean $\pm$ s.e.mean; $n=4$; $P<0.05$).

3 WEB 2086, a specific platelet-activating factor (PAF) receptor antagonist, induced a significant concentration-dependent decrease of such a lysis ($39.6 \pm 7.7\%$ protection at 100 μM; $n=4$). This potential role for PAF was confirmed with two other antagonists of this lipid mediator, i.e., BN 52021 and RP 48740.

4 Finally, we demonstrated that pretreatment of IVEC with WEB 2086 protected significantly against cell lysis induced by stimulated human neutrophils, an experimental model in which HLE participates.

Keywords: Elastase; endothelial cells; cytotoxicity; platelet-activating factor

Introduction

Polymorphonuclear neutrophils (PMN) play a crucial role in the development of ischaemia-reperfusion injury, a condition involved in myocardial infarction or in pulmonary diseases such as the adult respiratory distress syndrome, two pathological states characterized by a permeability oedema associated with the damage of endothelial cells (Warshawski *et al.*, 1986; Simon & Ward, 1988; Entman *et al.*, 1991). A better understanding of PMN-mediated endothelial cell injury has been made possible by use of endothelial cell cultures. Three PMN-derived products have been identified as cytotoxic mediators: proteinases, reactive oxygen products, as well as nitric oxide and its derivatives (Ward & Mulligan, 1993). It was demonstrated that endothelial cell detachment is mediated by PMN-derived proteinases which act by digesting proteins of the endothelial cell surface, including fibronectin (Harlan *et al.*, 1981). Other components of the basement membrane are affected by proteinases, and particularly by elastase (HLE), the major source of proteoglycan degrading activity in PMN (McGowan, 1990). By use of PMN from chronic granulomatous disease patients, this effect, which results in an increase of vascular permeability, was demonstrated to be oxygen radical-independent (Harlan *et al.*, 1985; Kaslovsky *et al.*, 1991). While it was well established that oxidants were of a minor importance as compared to proteinases in PMN-mediated disruption of endothelial cell monolayer, they were considered for a long time as the most cytolytic toxins released from PMN (Henson & Johnston, 1987; Weiss, 1989). Thus, PMN-mediated lethal cell injury was attributed specifically to oxygen products (Sacks *et al.*, 1978; Weiss *et al.*, 1981; Varani *et al.*, 1985). However, it is now recognized that PMN can also kill endothelial cells by a proteinase-dependent mechanism (Smedly *et al.*, 1986), a key role being attributed to HLE (Varani *et al.*, 1989; Ward & Mulligan, 1993).

The purpose of this study was to investigate the mechanism(s) responsible for HLE-induced human endothelial cell lysis (Okrent *et al.*, 1990; Chignard *et al.*, 1993). We determined whether platelet-activating factor (PAF), a lipid mediator synthesized by human endothelial cells incubated with HLE (Camussi *et al.*, 1988), was related to HLE-mediated endothelial cell damage. In fact, using specific inhibitors, we demonstrated that such a pathway was likely to intervene. In a second step, PAF receptor antagonists were tested against IVEC lysis induced by PMN.

Methods

Endothelial cells

Endothelial cells obtained from human umbilical cord vein were micro-injected with a recombinant DNA fragment composed of a deletion mutant of the human vimentin regulatory region controlling the SV40 early encoding sequence (Schwartz *et al.*, 1991). This immortalized venous human endothelial cell line (IVEC) has retained numerous differentiated properties of primary cultures as shown by morphological studies (light and electron microscopy), their capacity to secrete prostacyclin upon stimulation, and the detection of von Willebrand factor, as well as angiotensin converting enzyme and neutral endopeptidase activities (Llorens-Cortes *et al.*, 1992; Vicart *et al.*, 1993).

Cells were cultured at 37°C in 5% CO_2 atmosphere into 0.2% gelatin-coated 25 cm^2 or 75 cm^2 flasks (Falcon, Becton Dickinson Labware, Oxnard, U.S.A.). The medium used was the minimum essential medium with Earle's salts and L-glutamine (MEM, Gibco, Life Technology Ltd, Paisley, U.K.) supplemented with 10% heat-inactivated foetal calf serum (FCS; Boehringer Mannheim, Mannheim, Germany), penicillin (100 u ml^{-1}), streptomycin (100 $\mu g\ ml^{-1}$) and amphotericin B (0.25 $\mu g\ ml^{-1}$). Cells grown to confluency were subcultured by a brief trypsinisation (0.05% trypsin-0.02% EDTA prepared in modified Puck's saline A, Gibco, Life Technology Ltd, Paisley, U.K.). For stimulation, cells were seeded at a density of 50,000 per well in 24-well culture plates

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(Falcon, Becton Dickinson Labware, Oxnard, U.S.A.) so that they were confluent 3 days later. Cells in passages 20 to 34 were used in this study.

Neutrophils

PMN were isolated from blood of human volunteers as previously described (Renesto & Chignard, 1993). The final pellet was suspended in a volume of BME (BME with Earle's salts and L-glutamine, Gibco, Life Technology Ltd, Paisley, UK) such that the cell concentration was 6×10^6 PMN ml^{-1}.

Cytotoxicity assays

Confluent cultures of IVEC were incubated with sodium chromate ($Na^{51}CrO_4$, 2 μCi ml^{-1} of MEM + 10% FCS) for 18 h. Cells were then rinsed twice with BME and incubated in 500 μl fresh medium supplemented or without HLE or with 500 μl PMN (3×10^6 cells). At the end of the incubation period, medium was collected and wells washed with 500 μl BME. The washing media were pooled with corresponding first fractions (tubes A = lysed and detached cells). To discern cell lysis from detachment, samples were centrifuged (300 *g*; 10 min) and 500 μl were carefully collected (tubes B = half volume of lysed cells). Cells remaining in wells were lysed with 500 μl NaOH (25 mM) and SDS (0.1%) (tubes C = adherent cells). All tubes were counted in a gamma counter (LKB, Wallac, Stockholm, Sweden). The specific damage was determined by the following equations: lysis (in %) = $100 \times$ [lysis of test sample $(2 \times B)_{test}$ − spontaneous lysis $(2 \times B)_{control}$]/[remaining adherent cells in control $(C)_{control}$]. Detachment (in %) = $100 \times$ [detachment of test sample $(A - B)_{test}$ − spontaneous detachment $(A - B)_{control}$]/[remaining adherent cells in control $(C)_{control}$]. Each point was run in duplicate. Spontaneous ^{51}Cr release corresponding to control conditions was $11.2 \pm 0.6\%$, $17.2 \pm 1.1\%$, and $30.6 \pm 1.4\%$ at 3, 6 and 16 h, respectively. For spontaneous detachment, these values were $2 \pm 0.5\%$, $2.5 \pm 0.9\%$ and $4.2 \pm 0.9\%$, respectively.

Determination of HLE enzymatic activity

Enzymatic activity of HLE was determined spectrophotometrically by following hydrolysis of its specific synthetic substrate, N-succinyl-Ala-Ala-Ala-*p*-nitroanilide. Thus, 10 μg ml^{-1} HLE was incubated at 37°C in BME and in the presence or absence of WEB 2086 (200 μM), BN 52021 (50 μM) or RP 48470 (1 mM). Following different preincubation periods, the HLE substrate (1 mM final concentration) was added and formation of paranitroaniline residues was monitored at 410 nm every 20 s for 5 min. Results were expressed in variation of optical density $(\Delta OD) \times 10^{-3}$ min^{-1}.

Materials

HLE was purified from human leukocytes as previously described (Renesto *et al.*, 1990), following the method of Baugh & Travis (1976) modified by Martodam *et al.* (1978). For some experiments, catalytic sites of this proteinase were blocked by treatment with phenyl-methylsulphonyl fluoride (PMSF) (Renesto & Chignard, 1993). $Na^{51}CrO_4$ was from Amersham, Buckinghamshire, U.K. Gelatin (Type B from bovine skin), PMSF, N-formyl-Met-Leu-Phe (fMLP) and N-succinyl-Ala-Ala-Ala-*p*-nitroanilide were from Sigma Chemical Corp., St-Louis, Mo, U.S.A. WEB 2086 (3-[4-)2- chlorophenyl-9-methyl-6H-thieno[3,2-f][1,2,4] triazolo-[4,3-a](1,4]-diazepin-2-yl]-1-(4-morpholinyl)-1-propanon) was a gift from Dr H Heuer, Boehringer Ingelheim, Ingelheim, Germany. BN 52021 (3-(1,1-dimethylethyl) hexahydro-1,4,7b-trihydroxy-8-methyl-9H-1,7α (epoxymethano)-1H, 6αH-cyclopenta [c] furo(2, 3b) [3′,2′:3,4)cyclopenta(1,2-d]furan-5,9,12 (4H)-trione)) was a gift from Dr P Braquet, Institut Henri Beaufour, Le Plessis-Robinson, France and the compound RP 48740 (3-(3-pyridyl)-1H,3H-pyrrolo [1,2-c] thiazole-7 carboxamide) was a gift from Dr P. Sevidy, Rhône-Poulenc, Vitry, France.

Statistical analysis

All results were expressed as mean ± s.e.mean of at least 3 distinct experiments. The data were analyzed by Student's unpaired *t* test to determine whether differences were statistically significant. (*indicates $P < 0.05$).

Results

Endothelial cell injury induced by HLE

As observed with primary cultures of endothelial cells (Chignard *et al.*, 1993), exposure of IVEC to HLE induced a rapid cell retraction (not illustrated). This effect preceded cell injury since, under these conditions (10 μg ml^{-1} HLE for 1 h), neither significant cell detachment nor lysis occurred ($2.2 \pm 0.7\%$ and $1.5 \pm 0.6\%$, respectively, $n = 4$; $P > 0.05$).

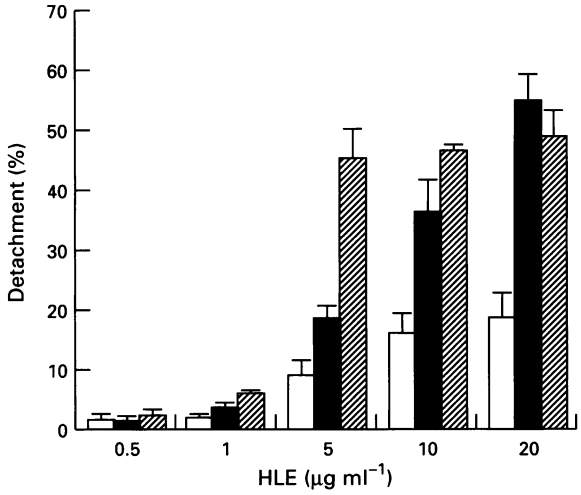

Figure 1 IVEC detachment induced by human leukocyte elastase (HLE). Labelled ^{51}Cr IVEC were overlaid with 500 μl BME containing increasing concentrations of HLE. After various incubation time periods, i.e. 3 h (open columns), 6 h (hatched columns) or 16 h (stippled columns), the percentage of detached cells was evaluated as described in Methods. Each column is the mean ± s.e.mean of 3–4 distinct experiments.

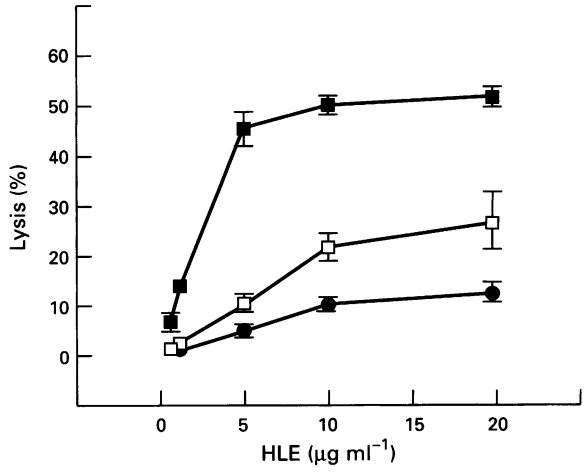

Figure 2 IVEC lysis induced by human leukocyte elastase (HLE): details as in Figure 1 with incubation time periods of 3 h (●), 6 h (□) and 16 h (■).

When cells were incubated with this proteinase for longer time intervals, significant concentration- and time-dependent cell detachment (Figure 1) and lysis (Figure 2) were observed. The intensity of the observed effects was strictly similar when evaluated by chromium assays or LDH measurements (not shown). This injurious effect of HLE was related to its enzymatic activity and was not a charge phenomenon, as demonstrated for other HLE-mediated cellular effects (LeRoy *et al.*, 1984; Peterson *et al.*, 1987). Indeed, incubation of IVEC with 10 $\mu g\ ml^{-1}$ PMSF-treated HLE for 6 h failed to affect cell integrity ($1.6 \pm 3.9\%$ detachment and $1.3 \pm 1.7\%$ lysis; $n=3$; $P>0.05$).

Protective effects of PAF antagonists on HLE-mediated IVEC lysis

As shown in Figure 3, when IVEC were exposed for 6 h to 10 $\mu g\ ml^{-1}$ HLE in presence of the specific synthetic PAF receptor antagonist, compound WEB 2086 (Casals-Stenzel, 1987), a concentration-dependent inhibition of cell lysis was observed. This effect was significant for 5 μM WEB 2086 and reached a plateau above 100 μM, the optimal concentration which can be used without damaging the cells themselves. Under these circumstances, cell detachment was not affected (data not shown).

In order to confirm a potential role of PAF in HLE-induced IVEC injury, two other PAF antagonists were tested, namely the compounds RP 48740 (Sevidy *et al.*, 1985) and BN 52021 (Braquet *et al.*, 1985). Our results showed that RP 48740 (1 mM) and BN 52021 (50 μM) also reduced cell lysis by $30.7 \pm 3.5\%$ and $25.8 \pm 6.4\%$, respectively ($n=3$; $P<0.05$). Since some PAF receptor antagonists are endowed with antiproteinase activity, such a possibility was investigated. HLE (10 $\mu g\ ml^{-1}$) was incubated at 37°C in the medium used for cytotoxicity assays in the presence or absence of inhibitors. At concentrations effective against lysis, none of the PAF antagonists affected the enzymatic activity of HLE (data not shown).

Effect of WEB 2086 on PMN-mediated IVEC lysis

Since PMN-induced endothelial cell injury is mediated in large part by HLE (Smedly *et al.*, 1986), we investigated the effect of the compound WEB 2086 on IVEC lysis caused by fMLP-stimulated PMN. First, we confirmed that HLE released from PMN indeed damaged IVEC. Incubation of the cells for 20 h with PMN stimulated by fMLP (2 μM) resulted in $19 \pm 2.6\%$ lysis ($n=7$). In contrast, when the same experiments were performed in the presence of eglin C (10 μM), an inhibitor of HLE (Schnebli *et al.*, 1985), lysis was only $8.6 \pm 1.6\%$ ($n=4$; $P<0.05$), thus confirming the participation of HLE in PMN-induced IVEC injury. Since adhesion of PMN to endothelial cells is influenced by PAF (Lorant *et al.*, 1991; Watanabe *et al.*, 1991), the presence of a PAF antagonist in the medium might inhibit indirectly the cytotoxicity by preventing in part adhesion, and presumably PMN degranulation. To counteract such an effect, the protocol previously used was modified. Thus, ^{51}Cr-labelled IVEC were preincubated for 5 h in the presence of 200 μM WEB 2086. The medium was then removed and cells washed before the addition of PMN. As seen in Figure 4, under these conditions, WEB 2086 was effective in reducing lysis induced by FMLP-stimulated PMN. As a control, purified HLE-induced lysis was also inhibited.

Discussion

Experiments described in this paper provide the first evidence that PAF intervenes in endothelial cell lysis induced by HLE. Such a conclusion was reached from the observation that WEB 2086, a specific PAF receptor antagonist, was able to prevent significantly lysis of IVEC exposed to this serine proteinase. Similar results were obtained with two other inhibitors with the same specificity of action, namely the compounds RP 48740 (Sevidy *et al.*, 1985) and BN 52021 (Braquet *et al.*, 1987). Moreover, we verified that these inhibitors failed to reduce the enzymatic activity of HLE. These data, and the fact that these three compounds are structurally distinct, ruled out a non-specific effect of PAF antagonists, As HLE induces PAF formation by endothelial cells (Camussi *et al.*, 1988), an effective role for PAF in HLE-mediated IVEC lysis can be inferred from the present data. Because HLE was known to play a preponderant role in the cytotoxic effect of PMN (Ward & Mulligan, 1993), the efficacy of WEB 2086 was then assayed against IVEC lysis induced by fMLP-stimulated PMN. As expected, our results indicated that PAF was also involved in PMN-mediated lysis of endothelial cells.

The interaction of HLE with its specific receptor recently identified on human endothelial cells (Abe *et al.*, 1993) may be

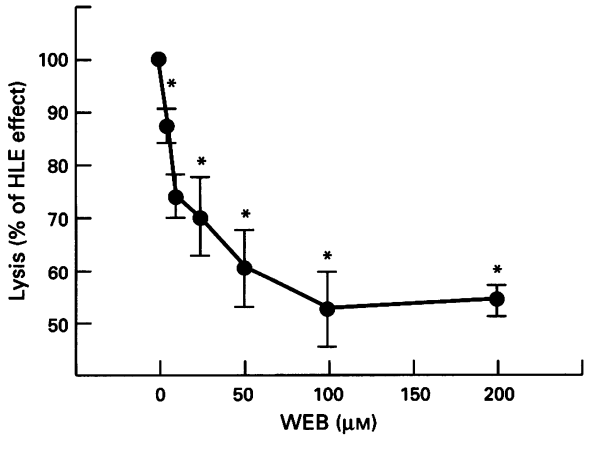

Figure 3 Protection by WEB 2086 of human leukocyte elastase (HLE)-induced IVEC lysis. Cells were preincubated with increasing concentrations of WEB 2086. One hour later, HLE (10 $\mu g\ ml^{-1}$) was directly added to the wells. Following a 6 h incubation, the reaction was stopped and samples were treated as described in Methods. Values are expressed as percentage of effect compared with control (lysis induced by HLE alone). Each point is the mean ± s.e.mean of 3–4 distinct experiments.

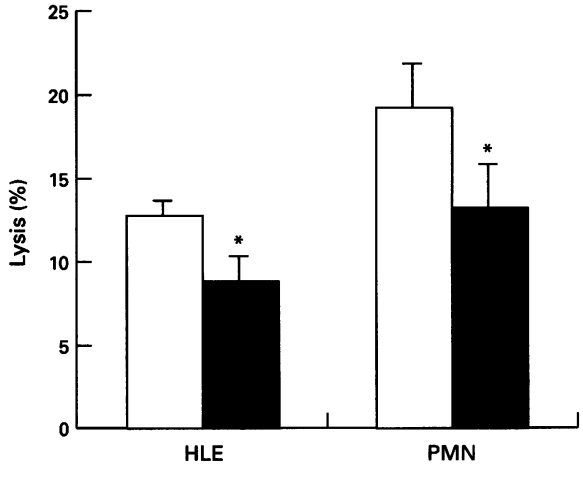

Figure 4 Pretreatment of IVEC with WEB 2086 inhibited human leukocyte elastase (HLE)- and activated PMN-induced lysis. Cells were preincubated for 5 h with 200 μM WEB 2086 (solid column) or not (control conditions, open columns). Medium was then aspirated and cells washed before addition of either HLE (1 $\mu g\ ml^{-1}$) or PMN (3×10^6 cells/well) stimulated with fMLP (2 μM). The reaction was stopped 20 h later and samples were treated as described in Methods. Results were expressed as percentage of lysed cells, calculated as described in Methods. Each column is the mean ± s.e.mean of 3–6 distinct experiments.

the initial event in the killing process. Another seductive route for PAF synthesis by HLE was suggested by Camussi *et al.* (1988) and concerned the cleavage of lipocortin which would promote the activation of phospholipase A_2. Such an effect implicates the entry of HLE within cells, a phenomenon which seems to be effective (Ward & Mulligan, 1993). In endothelial cells, the majority of PAF synthesized upon stimulation remains intracellular (Henson, 1987). This observation suggested a role for PAF within the cell through interaction with non-identified intracellular receptors (Shukla, 1992). In the experimental conditions described in the last figure, the efficacy of WEB 2086 preincubated with IVEC and removed during HLE or PMN exposure indeed suggests an interaction with an intracellular PAF receptor. Accordingly, it may be hypothesized that exposure of IVEC to HLE induces synthesis of PAF which, in turn, elicits endothelial cell lysis.

While we consider that underlying mechanisms accounting for the HLE effect merit further study, the finding that PAF intervenes represents, *per se*, a crucial point, all the more since WEB 2086 efficiency has been extended to the lysis mediated by FMLP-activated PMN. PAF may be involved in various physiopathological states (Braquet *et al.*, 1987), including adult respiratory distress syndrome (Braquet & Hosford, 1989). In fact, the participation of this mediator in human pathologies and animal models of tissue injury is largely based on the effectiveness of PAF antagonists, although its precise mechanism of action is not fully understood (Braquet *et al.*, 1987). For example, Warren *et al.* (1989) have shown that the PAF receptor antagonist, L-652,731, provided a significant degree of protection against the neutrophil-dependent vascular injury induced by immune complexes, although, and unexpectedly, such an effect was not accompanied by a reduction of PMN accumulation in lung tissue. As a consequence, it was hypothesized that PAF acts as a primer for PMN, enhancing their oxygen metabolite production and proteinase release in response to immune complexes. The findings presented in this paper could provide another alternative pathway by which PAF could intervene in vascular injury.

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