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Drug-induced *in vitro* inhibition of neutrophil-endothelial cell adhesion

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1 Leukocyte-endothelial cell interactions play an important role during ischaemia-reperfusion events. Adhesion molecules are specifically implicated in this interaction process.

2 Since defibrotide has been shown to be an efficient drug in reducing damage due to ischaemia-reperfusion in many experimental models, we analysed the effect of defibrotide *in vitro* on leukocyte adhesion to endothelial cells in basal conditions and after their stimulation.

3 In basal conditions, defibrotide (1000 μg ml^{-1}) partially inhibited leukocyte adhesion to endothelial cells by 17.3% $\pm$ 3.6 ($P<0.05$), and after endothelial cell stimulation (TNF-α, 500 u ml^{-1}) or after leukocyte stimulation (fMLP, 10^{-7} M), it inhibited leukocyte adhesion by 26.5% $\pm$ 3.4 and 32.4% $\pm$ 1.8, respectively ($P<0.05$).

4 In adhesion blockage experiments, the use of the monoclonal anntibody anti-CD31 (5 μg ml^{-1}) did not demonstrate a significant inhibitory effect whereas use of the monoclonal antibody anti-LFA-1 (5 μg ml^{-1}) significantly interfered with the effect of defibrotide.

5 This result was confirmed in NIH/3T3-ICAM-1 transfected cells.

6 We conclude that defibrotide is able to interfere with leukocyte adhesion to endothelial cells mainly in activated conditions and that the ICAM-1/LFA-1 adhesion system is involved in the defibrotide mechanism of action.

Keywords: Chemoattractant; cytokines; defibrotide; integrins

Introduction

Alterations in endothelial cell function occur as a result of ischaemia-reperfusion processes. An important role in the pathogenesis of damage due to ischaemia-reperfusion has been attributed to free-radical production. In fact, the reactive oxygen species are responsible for alterations in endothelial membrane permeability, as measured by decrease in protein, glyceride and phospholipid synthase, with concurrent increases in lipid peroxidation and cholesterol synthesis (Kozar *et al.*, 1994). Reactive oxygen species are the products of phagocytic activity of polymorphonuclear leukocytes, monocytes and macrophages and of cellular respiration. Lymphocytes, which do not exert phagocytic activity, can also generate oxygen species during cellular respiration. Any antigenic or regulatory stimulus of their cell activity will increase the expression of oxygen derivatives (O_2^-, $OH^\bullet$, H_2O_2) (Buttke & Sandstrom, 1994). Leukocyte adhesion to endothelial cells is the first of a multistep process that leads to vascular infiltration and damage by leukocytes during ischaemia-reperfusion episodes (Kukielka *et al.*, 1993). Adhesion molecules play an important role in such processes (Springer, 1990).

Oxygen-free radicals are stimuli useful in activating endothelial cells as well as histamine, thrombin, bradykinin and leukotriene C_4 (Lefer *et al.*, 1991). Such an activation due to oxygen-free radicals induces the expression of adhesion molecules on some circulating cells (especially neutrophils and platelets) and on endothelial cells, such as P-selectin. E-selectin, expressed solely on endothelial cells, is synthesized rapidly after stimulation by tumour necrosis factor-α (TNF-α) and interleukin-1 (IL-1). Selectin-dependent adhesion of leukocytes to endothelium, which precedes their transendothelial migration (diapedesis), requires the activation of other adhesion molecules (such as integrins and members of the immunoglobulin superfamily) to permit the passage from leukocyte 'rolling' to strong adhesion (Mackay & Imhof, 1993).

Intercellular adhesion molecule-1 (ICAM-1, an immunoglobulin located on endothelial cells and lymphocytes) is the ligand for lymphocyte function-associated molecule-1 (LFA-1, an integrin located on polymorphonucleates, monocytes and lymphocytes). Like other integrins, LFA-1 increases its adhesiveness towards its counter receptor (ICAM-1) after stimulation by different agents such as formyl-methionyl-leucyl-phenylalanine (fMLP). Platelet endothelial cell adhesion molecule-1 (PECAM-1 or CD31) is an immunoglobulin located on leukocytes and on endothelial cells and is required for the transmigration of neutrophils across endothelial monolayers (Vaporciyan *et al.*, 1993). fMLP is a chemotactic factor that can also augment leukocyte adhesion to the endothelium (Lefer *et al.*, 1991).

We previously limited tissue damage due to ischaemia-reperfusion by using the drug defibrotide (DF) in many experimental models of organ ischaemia in rats. DF is a single-stranded DNA fraction with profibrinolytic, antithrombotic and thrombolytic properties (Pescador *et al.*, 1983; Niada *et al.*, 1986), and it can trigger the release of prostacyclin (PGI_2) from endothelium (Gryglewski *et al.*, 1989). We have demonstrated that DF administration protected the metabolic activity of ischaemic heart (Ferrero *et al.*, 1989; 1990b), kidney (Ferrero *et al.*, 1990b; Marni *et al.*, 1990; Corsi *et al.*, 1993b) and liver of rat (Ferrero *et al.*, 1990b, c).

Protection of rat heart and kidney from damage due to ischaemia-reperfusion during procurement and grafting by DF has also been demonstrated in many experimental models and at different times following transplantation (Ferrero *et al.*,

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1990a; 1991a, b; 1993a, b; Corsi *et al.*, 1993c). In view of such results, we supposed that DF could protect endothelial cells from ischaemic damage, thus favouring their function, in the same manner as the drug protected ischaemic tissues from metabolic damage measured by the drop of adenosine triphosphate and NAD^+. Indeed, we hypothesized that DF could be considered a direct oxygen-free-radical scavenger or possibly that its ability to limit oxygen intermediate generation is linked to its capability in PGI_2 generation, which exerts a 'cytoprotective' action in ischaemic organs (Ferrero *et al.*, 1988; 1991c).

We thus studied the capacity of DF to regulate leukocyte adhesion to endothelial cells. We used an *in vitro* experimental model by which we tested the adhesion of granulocytes to endothelium in basal conditions or in the presence of factors useful for modifying such adhesion. In fact, a possible inhibition of such a process, DF-induced, could control or reduce postischaemic damage due to oxygen-free radical production, which is dependent on leukocyte and endothelial cell activation.

Methods

Endothelial cell cultures

NIH/3T3-ICAM-1 transfected cells were kindly procured by Dr. R. Pardi. The permanent human endothelial cell line EA.hy 926 was kindly provided by Dr Cora Edgell. EA.hy 926 cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco, Paisley, U.K.) supplemented with 10% foetal calf serum (FCS, Flow, Irvine, Scotland), gentamicin (50 $\mu g\ ml^{-1}$, Gibco) and nystatin (40 u ml^{-1}, Gibco). This human endothelial cell line has been shown to express factor VIII-related antigens with the same morphological distribution as in primary human endothelial cells. Primary cultures of human umbilical vein endothelial cells (HUVEC) were derived from umbilical cords and maintained as described (Jaffe, 1984). HUVEC were found to express endothelial-related antigens such as PECAM and factor VIII (data not shown). For the adhesion assay, endothelial cells, detached by a brief exposure to 0.25% trypsin and 0.22% EDTA, were plated and grown to confluence in 24-well plates.

Measurement of granulocyte adhesion to endothelial cells

Granulocytes (PMN), prepared as described subsequently, were radiolabelled for 1 h at room temperature with $Na_2{}^{51}CrO_4$ (1 mCi/10^6 cells, Amersham, Milan, Italy), washed twice, and resuspended at $5 \times 10^6\ ml^{-1}$ in HEPES-Tyrode buffer. Their viability was assessed by the Trypan blue dye exclusion test. Radiolabelled PMN suspensions (100 μl) were layered on endothelial cells in the presence or absence of DF and incubated for 15 min at 37°C. In resting conditions, neutrophil adhesion was about 6% of added neutrophils, and neutrophil radioactivity was about 0.05 c.p.m./neutrophil. The loss of radioactivity from neutrophils during the course of the experiment was <5%. In our experiments, treatments were performed at four different times: overnight, 4 h, 1 h and 1 min before leukocyte addition to monolayers of endothelial cells. Before the 1 h treatment, each well was washed three times with phosphate-buffered saline (PBS). Leukocyte activation was obtained by preincubation (3 min) with 10^{-7} M fMLP, whereas endothelial cell activation was obtained by an overnight treatment with 500 u ml^{-1} TNF-α (Endogen, Boston, MA, U.S.A.). An NO synthase inhibitor, N^{ω}-nitro-L-arginine methyl ester (L-NAME, 10^{-5} M, Sigma-Aldrich, Milan, Italy), was also used in some experiments. In adhesion blocked experiments, leukocytes were preincubated with monoclonal $F(ab)_2$ antibodies (anti-LFA-1 [70H12] and anti-CD31 [M89D3], 5 $\mu g\ ml^{-1}$ for 20 min at 4°C. The anti-LFA-1 α 70H12 monoclonal antibody was kindly provided by Dr A. Poggi (Laboratory of Immunopathology, Italian National Institute for Cancer Research, Genoa). The specificity for the α chain was assessed using LFA-1 α-expressing Cos-7 transfected cells (data not shown). The antibody was purified by affinity chromatography and the $F(ab)_2$ fragment prepared by pepsin digestion according to Parham (1983). At the end of the incubation time, the wells were washed three times to remove nonadherent cells, the remaining bound cells were lysed with Triton X-100 (1%, BDH, Poole, U.K.), and the individual lysates were counted in a gamma counter (model 5000, Packard, Sterling, VA, U.S.A.).

ICAM-1 transfectants

ICAM-1^+-transfected cells were obtained from NIH/3T3 murine fibroblasts with ICAM-1 and neomycin resistance on the same plasmid. ICAM-1 subcloned into pcDNA I/Neo (Invitrogen, San Diego, CA, U.S.A.) at the XbaI site was kindly provided by Dr Ruggero Pardi (Human Immunology Unit, Scientific Institute San Raffaele, Milan, Italy). Transfection was performed by calcium phosphate-DNA coprecipitation. Stable transfectants were selected by addition of the neomycin analogue G418 to a final concentration of 0.8 mg ml^{-1}. Neomycin-resistant colonies were picked 10 days later, expanded and tested for ICAM-1 expression by immunofluorescence using the 84H10-I0154 monoclonal antibody (Immunotech, Luminy-Marseille, France).

Pharmacological treatment

DF was a gift from Crinos Biological Research Laboratories (Villa Guardia, Como, Italy). The drug is a polydeoxyribonucleotide obtained by controlled hydrolysis of DNA from mammalian lungs, with a molecular weight of approximately 20 kD. DF was added to endothelial cells to give a concentration of 1000 $\mu g\ ml^{-1}$ of medium, which was shown to be the maximal inhibitory dose for endothelial-neutrophil adhesion. The DF doses studied were 100, 500 and 1000 μg, and the latter was the maximal inhibiting dose.

Preparation of PMN

Venous blood from healthy donors who had not received any medication for at least 2 weeks was anticoagulated with 0.065 M citric acid (Riedel, Hannover, Germany), 0.085 M sodium citrate (Farmitalia, Milan, Italy), and 2% glucose monohydrate (Riedel) in a blood:anticoagulant ratio of 7:1. PMN were isolated by dextran (Sigma, Milan, Italy) sedimentation followed by Lymphoprep (Nycomed, Oslo, Norway) gradient and hypotonic lysis of erythrocytes (Boyum, 1968). PMN were washed with PBS without calcium and magnesium (Gibco) and resuspended in ice-cold HEPES-Tyrode buffer (pH 7.4) containing (mM): NaCl 129, $NaHCO_3$ 9.9, KCl 2.8, KH_2PO_4 0.8, $MgCl_2 \cdot 6H_2O$ 0.8, glucose monohydrate 5.6, $CaCl_2$ 1 and HEPES 10. Cell suspensions contained more than 97% viable PMN, as evaluated by the Trypan blue dye exclusion test. PMN were used within 2 h of their isolation. Cell suspensions contained 95% PMN; an average of 1 platelet per 10 to 20 PMN was usually observed.

Statistical analysis

Statistical analyses were performed with the Mann-Whitney and Wilcoxon tests for nonparametric results, and the results were considered statistically significant when $P<0.05$.

Results

In our work, we analysed the effects of DF on neutrophil adhesion to endothelial cells in basal conditions and after endothelial or leukocyte activation. Endothelial cell stimulation was obtained with 500 u ml^{-1} TNF-α in an overnight treatment, and leukocyte activation was obtained by addition of

10^{-7} M fMLP to leukocytes 3 min before their addition to endothelial cell monolayers. The data are expressed as ^{51}Cr measured in percentage with respect to controls. In preliminary dose-response experiments, 1000 $\mu g\ ml^{-1}$ was found to be the most efficient DF concentration (Figure 1).

Effect of DF on granulocyte adhesion to endothelial cells in basal conditions and in the presence of TNF-α

In basal conditions, DF slightly but significantly reduced leukocyte adhesion to EA.hy 926 endothelial cells after only 1 min of treatment (17.3% ± 3.6; $P<0.05$) (Figure 2). The same results were obtained when HUVEC were used (13% ± 2.8; $P<0.05$; data not shown).

TNF-α increased neutrophil adhesion to EA.hy 926 (386.7% ± 45.9 vs. controls) (Figure 2) and to HUVEC (248.5% ± 25.1) (data not shown). After endothelial cell stimulation (TNF-α overnight), DF significantly inhibited neutrophil adhesion after an overnight as well as a 1 min treatment (33% ± 4.9 and 26.5% ± 3.4, respectively; $P<0.05$) (Figure 2). To evaluate the role of endothelial cell NO in DF inhibition on TNF-induced leukocyte adhesion, an NO synthase inhibitor was used. DF exerted the same level of inhibition in the presence and absence of L-NAME (10^{-5} M overnight) (data not shown). Such data indicated that DF inhibition was prevalent in leukocytes but also present in endothelial cells. To test this hypothesis, we analyzed the capacity of DF to inhibit leukocyte activation by 10^{-7} M fMLP.

Effect of DF on fMLP granulocyte-activated adhesion to endothelial cells

Using 10^{-7} M fMLP, maximal leukocyte adhesion (173.3% ± 8.1 vs. controls) was obtained after about 15 min of leukocyte-endothelial cell incubation. Indeed, different incubation times were analyzed (15, 30 and 60 min), but the highest leukocyte adhesion was obtained after 15 min. After fMLP stimulation, DF (added 1 min before fMLP) inhibited leukocyte adhesion to endothelial cells (32.4% ± 1.8; $P<0.05$) (Figure 3).

Effect of monoclonal F(ab')$_2$ antibodies anti-LFA-1 and anti-CD31 on neutrophil adhesion to endothelial cells: influence of DF

Monoclonal antibodies directed toward CD31 and LFA-1 adhesion molecules were used. With anti-CD31, adhesion of leukocytes was not inhibited in basal conditions or after fMLP stimulation. Furthermore, in the presence of the antibody, DF exerted its inhibitory action (data not shown). When anti-LFA-1 was used, neutrophil adhesion was inhibited in basal conditions and after fMLP stimulation (56% ± 4.8 and 66.5% ± 5.5, respectively; $P<0.05$). In this experimental situation, DF exerted only slightly greater inhibition when fMLP was added (24% ± 4.9; $P<0.05$) (Figure 4).

Effect of DF on neutrophil adhesion to NIH/3T3-ICAM-1 transfected cells

The results obtained indicated that leukocyte integrins were the main adhesion molecule system involved in DF inhibition processes and that the ICAM-1/LFA-1 complex was the

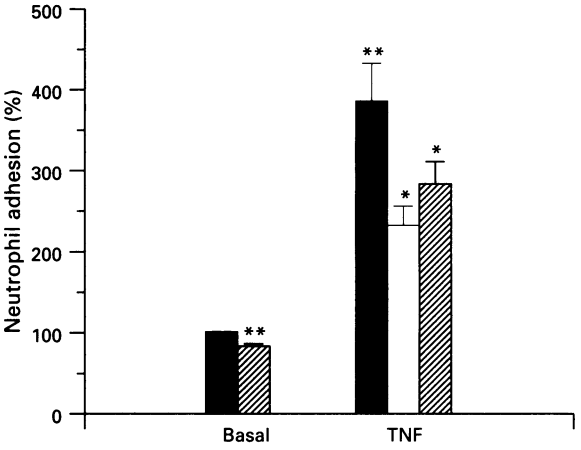

Figure 2 Effect of DF (1000 $\mu g\ ml^{-1}$) on neutrophil adhesion to EA.hy 926 cells. The data represent leukocyte adhesion in basal conditions and after endothelial cell stimulation (with TNF-α, 500 u ml^{-1} overnight) in the absence (solid column) or presence of DF for 1 min (hatched column) or overnight (open column). The values are expressed as the mean ± s.e.mean of four experiments, each done in triplicate. *$P<0.05$ vs. TNF without DF. **$P<0.05$ vs. basal without DF.

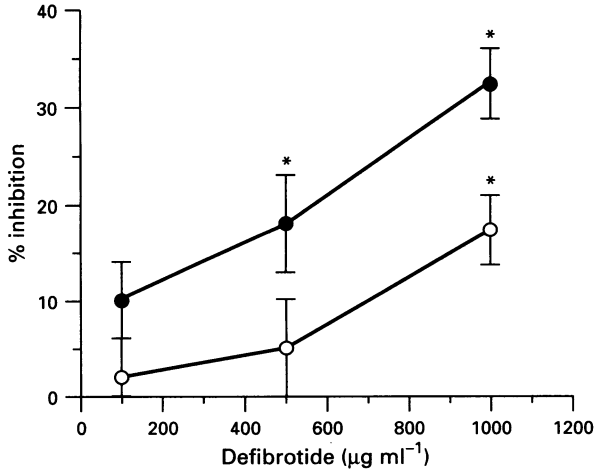

Figure 1 Inhibition (%) caused by different doses (100, 500 and 1000 $\mu g\ ml^{-1}$) of DF on neutrophil adhesion to EA.hy 926 cells. DF in basal conditions (○) or after fMLP stimulation (●) was added for 1 min to endothelial cells. The values represent the mean ± s.e.mean of four experiments, each done in triplicate. *$P<0.05$ vs. control.

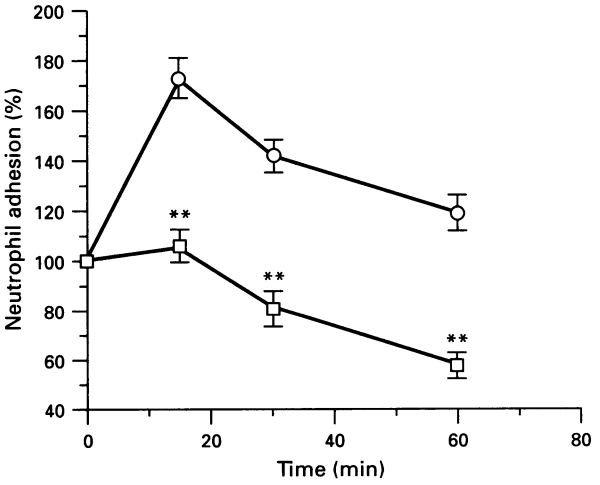

Figure 3 The effect of DF on the adhesion of fMLP-activated neutrophils to endothelial cells. fMLP (10^{-7} M) (○) and fMLP (10^{-7} M) plus DF (1000 $\mu g\ ml^{-1}$) (□) were used at different endothelial cell-leukocyte incubation times (15, 30 and 60 min). fMLP and DF were added to the leukocyte suspensions 3 min and 1 min respectively before their addition to endothelial cell monolayers. The data are expressed as the mean ± s.e.mean of four experiments, each done in triplicate. **$P<0.05$ vs. fMLP.

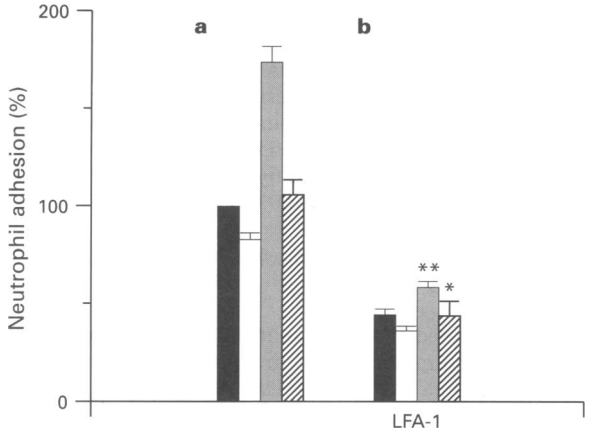

Figure 4 The effect of DF (1000 $\mu g\,ml^{-1}$) on the adhesion of resting and fMLP (10^{-7} M)-activated neutrophils to EA.hy 926 endothelial cells in the presence (b) or in the absence (a) of anti-LFA-1 monoclonal antibody. Controls (solid columns); DF (open columns); fMLP (stippled columns); fMLP+DF (hatched columns). When present, monoclonal antibody (Fab2, 5 $\mu g\,ml^{-1}$) was added to the leukocyte suspensions 20 min before their addition to the endothelial cell monolayers. The data are expressed as the mean ± s.e.mean of four experiments, each done in triplicate. $*P<0.05$ vs. fMLP. $**P<0.05$ vs. control.

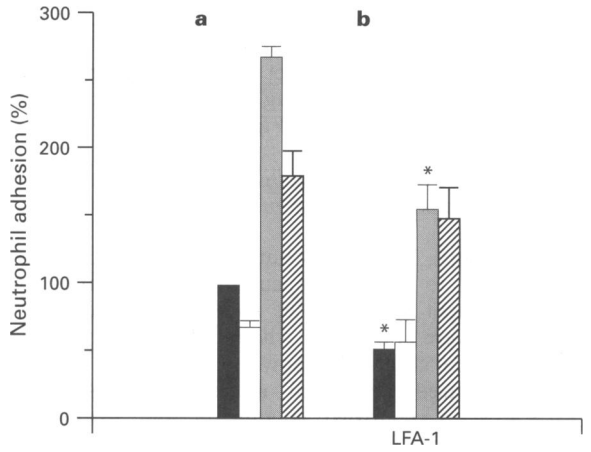

Figure 5 The effect of DF (1000 $\mu g\,ml^{-1}$) on the adhesion of resting or fMLP (10^{-7} M)-activated neutrophils to NIH/3T3-ICAM-1 transfected cells in the presence (b) or absence (a) of anti-LFA-1 monoclonal antibody (Fab2, 5 $\mu g\,ml^{-1}$). Controls (solid columns); DF (open columns); fMLP (stippled columns); fMLP+DF (hatched columns). Data are expressed as the mean ± s.e.mean of four experiments, each done in triplicate. $*P<0.05$ vs. same condition without anti-LFA-1.

principal target of its action. To test this hypothesis, ICAM-1 transfected NIH/3T3 cells were used. In this experimental condition, neutrophil adhesion was assured only by the ICAM-1/β2 integrin adhesion molecule system. Activation of leukocytes via 10^{-7} M fMLP increased adhesion to NIH/3T3-ICAM-1 transfected cells (267.1% ± 9.2 vs. controls). DF was able to inhibit neutrophil adhesion to NIH/3T3-ICAM-1 transfected cells in basal conditions and after addition of fMLP-activated leukocytes (30.9% ± 4.1 and 32.7% ± 6.1, respectively; $P<0.05$). When anti-LFA-1 monoclonal antibody was used, in basal conditions and in the presence of fMLP, the inhibition was 42.5% ± 25.1 and 52.7% ± 17.1, respectively ($P<0.05$). DF did not exert further significant inhibition, indicating that DF and anti-LFA-1 monoclonal antibody exerted their action on the same cell target (Figure 5).

Discussion

The role of oxygen-derived free radicals in postreperfusion endothelial dysfunction has been previously described after experimental myocardial ischaemia (Albelda *et al.*, 1994). The use of pharmacological agents useful in preserving endothelial integrity has been suggested: superoxide dismutase, PGI_2 and analogues, transforming growth factor β (which inhibits TNF production), and thromboxane synthetase inhibitors (Albelda *et al.*, 1994). Since the main source of oxygen-derived free radicals is phagocytic cells (such as neutrophils), blocking activation of the latter could possibly prevent such a production. Activation of leukocyte integrin molecules such as LFA-1 occurs in the inflammatory adhesion mechanism, in which neutrophils bind to endothelial cells. Many adhesion molecules are involved in the binding of cells to endothelium. The use of monoclonal antibodies against adhesion molecules, which prevents cell adhesion to endothelium, has been studied for potential application in the treatment of a variety of human diseases, such as inflammation (Górski, 1994).

We have used DF as an anti-ischaemic drug in many experimental models of heart, kidney and liver ischaemia-reperfusion and of heart and kidney transplantation in rats (Ferrero *et al.*, 1988; 1989; 1990a, b, c; 1991a, b, c; 1993a, b; Marni *et al.*, 1990; Corsi *et al.*, 1993b, c). Since DF has been shown to be able to increase the endogenous synthesis of PGI_2 from endothelial cells, it has been suggested as a PGI_2-mimetic (analogously to iloprost), with a beneficial action on stunned myocardium (Gross, 1993). PGI_2 has an inhibitory effect on oxygen-free-radical production by neutrophils, and the anti-ischaemic activity of DF could be explained by a stimulation of PGI_2 production.

However, we supposed a direct action of DF in limiting tissue damage due to ischaemia-reperfusion in consideration of the drug's ability to limit *in vivo* the production of lipoperoxides (our unpublished data) and to reduce leukocyte adherence in rats (Bianchi *et al.*, 1992). Since *in vivo* neutrophil binding to the microvascular endothelium (during reperfusion of an ischaemic organ) is the essential step for cellular activation and the production of free radicals (de la Ossa *et al.*, 1992), we studied the effect of DF on *in vitro* adhesion of granulocytes to endothelial cells. The DF concentration used was very high, but in accordance with concentrations used previously by us and by other authors *in vitro* and *in vivo*.

Using EA.hy 926 and HUVEC as endothelial cells, DF was able to inhibit leukocyte adhesion in basal conditions and after leukocyte or endothelial cell activation. In basal conditions, DF exerted a slight but significant inhibition only when added to endothelial cell monolayers 1 min before leukocyte addition. At the other incubation times, DF did not show any inhibitory activity. Such data suggest that in basal conditions the principal target of DF action is leukocytes.

Cytokines such as TNF-α are able to increase leukocyte adhesion to endothelial cells by increased or *de novo* expression of adhesion molecules (Pober *et al.*, 1986). Several adhesion molecules (selectins, immunoglobulins, cadherins and integrins) are involved in such processes. It is also known that NO produced by endothelial cells in basal conditions or after cytokine stimulation is able to modify leukocyte activity (Korbut *et al.*, 1989). When we used TNF-α we increased leukocyte adhesion to EA.hy 926 and HUVEC monolayers. DF was able to reduce TNF-α-induced leukocyte adhesion. Such data indicate that in this experimental situation, DF exerts its action on leukocytes as well as on activated endothelial cells. The fact that in basal conditions DF did not exert the same inhibitory activity on endothelial cells indicates that it interfered with the activation of cytokine-induced processes.

NO is not involved in the action of DF on activated endothelial cells. Indeed, use of an NO synthesis inhibitor (L-NAME, 10^{-5} M overnight) did not modify the effect of DF.

Neutrophil extravasation events involve several different adhesive proteins: central among these are the β2 integrins. Selection-mediated rolling is necessary but not sufficient for further β2-mediated adhesion. The latter requires activation of integrins, which on circulating leukocytes are in their inactive state. Activation can be accomplished by various inflammatory stimuli such as TNF-α, C5a, platelet-activating factor, or fMLP (Hynes, 1992). β2 integrin activation of leukocytes and their strong adhesion to counter-receptors (in particular, the ICAM-1/LFA-1 complex) represents a central event of the process.

To investigate the mechanism(s) of action of leukocytes by DF, a chemotactic agent, fMLP, was used. fMLP-treated leukocytes showed increased adhesion to endothelial cell monolayers. In such conditions DF completely annulled fMLP activation. It has been reported that activation of β2 integrins (such as LFA-1) occurs rapidly and reversibly in response to soluble stimuli (Dustin & Springer, 1989). Accordingly, in our study we obtained maximal activation at 15 min after fMLP stimulation, with a decrement thereafter.

CD31 belongs to the group of immunoglobulin adhesion molecules. It is involved in leukocyte adhesion to endothelial cells, and its expression on endothelial cells or leukocytes is regulated by different stimuli (e.g. TNF-α). It has also been reported that CD31 is able to amplify integrin-mediated adhesion of CD31-positive leukocytes to endothelial cells (Tanaka, 1992).

For a better comprehension of the mechanisms involved in the action of DF on leukocytes, adhesion-blockage experiments were performed with anti-CD31 and anti-LFA-1 $F(ab')_2$ monoclonal antibodies. In our experimental conditions, anti-CD31 did not exert a significant inhibitory effect on leukocyte adhesion to endothelial cells in basal conditions or after leukocyte activation. In the presence of anti-LFA-1, basal and stimulated adhesion was reduced. In this condition, DF induced only slightly greater inhibition. The data indicated that the ICAM-1/LFA-1 adhesion system could be involved in the inhibitory action of DF along with other adhesion molecules.

NIH/3T3-ICAM-1 transfected cells were used to confirm this hypothesis. Using this cell type, in which the only adhesion system involved was ICAM-1/β2 integrins (LFA-1, Mac-1, p150,95), DF was also able to exert its inhibitory effect in a manner similar to that obtained with anti-LFA-1. DF therefore inhibited leukocyte adhesion to endothelial or other cells by interfering with the ICAM-1/LFA-1 adhesion system.

Since activated leukocytes (such as those obtained with fMLP) enhance their adhesiveness by different mechanisms, including integrin activation (Jäättelä, 1991), it is possible that DF exerted part of its action by interfering with the intracellular processes that lead to integrin modification (phosphorylation and/or binding with cytoskeletal proteins) and subsequently with their activation.

Our results appear to be important in the explanation of our previously reported results. In fact, we obtained a prolongation in survival of grafted rat hearts by the use of subtherapeutic doses of cyclosporine and DF (Corsi *et al.*, 1993a). The data could be interpreted on the basis of an inhibition induced by the drug on neutrophil adhesion to endothelial cells following reperfusion due to organ transplantation. The drug's activity was possibly useful in inhibiting the involvement of neutrophils in the rejection process. In fact, successful use of the anti-LFA-1 monoclonal antibody in bone marrow transplantation (Fischer *et al.*, 1986) and in the prevention of early rejection of human renal allografts (Le Mauff *et al.*, 1991) is in accord with our results and our demonstration of a DF-induced inhibition of LFA-1.

In conclusion, the efficacy of DF as an anti-ischaemic drug could be related not only to its ability to increase PGI_2 production by endothelial cells, but also to its capability to affect LFA-1 activity by inhibiting neutrophil adhesion to endothelium.

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