ELSEVIER

European Journal of Pharmacology 318 (1996) 153–160

ejp

Leukocyte integrin very late antigen-4/vascular cell adhesion molecule-1 adhesion pathway in splanchnic artery occlusion shock

Francesco Squadrito [a,*], Domenica Altavilla [a], Letteria Ammendolia [a], Giovanni Squadrito [b], Giuseppe M. Campo [a], Patrizia Canale [a], Mariapatrizia Ioculano [a], Caterina Musolino [b], Andrea Alonci [b], Antonino Saitta [b], Francesco Rossi [c], Achille P. Caputi [a]

[a] *Institute of Pharmacology, School of Medicine, University of Messina, Piazza XX Settembre 4, 98121 Messina, Italy*
[b] *Department of Internal Medicine, School of Medicine, University of Messina, Messina, Italy*
[c] *Institute of Pharmacology, Second University of Naples, Naples, Italy*

Received 10 September 1996; accepted 13 September 1996

Abstract

We investigated the role played by the very late antigen-4 (VLA-4)/vascular cell adhesion molecule-1 (VCAM-1) interaction in the pathogenesis of splanchnic artery occlusion shock. Splanchnic artery occlusion shock was induced in anaesthetized rats by clamping splanchnic arteries for 45 min. Sham operated animals were used as controls. Survival time, serum tumour necrosis factor (TNF-α), monocyte and lymphocyte cell count and the responsiveness to acetylcholine of aortic rings were studied. Furthermore we investigated the VCAM-1 expression on vessel endothelium and the percentage of VLA-4 positive leukocytes. Splanchnic artery occlusion shocked rats had a decreased survival time (76 ± 10 min, while sham shocked rats survived more than 4 h), increased serum levels of TNF-α (328 ± 11 U/ml), a decreased number of both monocytes and lymphocytes and reduced responsiveness to acetylcholine (10 nM–10 μM) of aortic rings. In addition we found an increased expression of endothelial VCAM-1 on aortic rings and a reduced percentage of VLA-4 positive lymphocytes and monocytes. Passive immunization with specific antibodies raised against either VCAM-1 or VLA-4 (2 mg/kg, i.v., 3 h before splanchnic artery occlusion shock) increased survival, improved monocyte and lymphocyte count and restored the responsiveness of aortic rings to acetylcholine ($P < 0.01$). Finally, inhibition of TNF-α biosynthesis reversed the increased endothelial expression of VCAM-1 and the reduced percentage of integrin VLA-4 positive leukocytes. Our findings suggest that (i) VLA-4/VCAM-1 interaction has a role in the pathogenesis of circulatory shock; (ii) this interaction might be a target for new therapeutic approaches to the therapy of low-flow states.

Keywords: VLA-4 (very late antigen-4) integrin; VCAM-1 (vascular cell adhesion molecule-1); SAO (Splanchnic artery occlusion shock); (Rat)

1. Introduction

Leukocyte-endothelial interactions are regulated by a cascade of molecular steps that correspond to the morphological changes that accompany adhesion (Butcher, 1991; Lawrence and Springer, 1991; Smith et al., 1991).

This adhesion cascade can be divided into four sequential steps of rolling, triggering, strong adhesion and migration. Rolling is mediated by a family of three lectin-like carbohydrate-binding molecules called selectins each of which promotes leukocyte rolling under flow conditions (Zimmerman et al., 1992; Shimizu et al., 1992).

Each selectin recognizes specific carbohydrate sequences on either leukocytes (L-selectin or P-selectin) or the endothelium (E-selectin) and thereby mediates rolling of particular subsets of leukocytes.

A triggering step is required to promote strong adhesion that can stop the rolling leukocyte. One important trigger factor is the chemotactic cytokine interleukin-8, a chemokine that is produced either by the endothelium itself or by underlying inflammatory cells. Interleukin-8 can be retained on endothelium where it acts as an immobilized ligand for the rolling leukocyte (Rot, 1992; Hubere et al., 1991). Besides interleukin-8, other chemokines, notably macrophage inflammatory protein 1α (MIP-1α) and MIP-1β may serve as triggering factors (Taub et al., 1993; Schall, 1991).

* Corresponding author. Tel.: (39-90) 712-533; Fax: (39-90) 661-029.

'Strong adhesion' is mediated by leukocyte integrins that bind to counter receptors on endothelium. The β1 integrin VLA-4 mediates lymphocyte and monocyte binding to the endothelial vascular cell adhesion molecule-1 (VCAM-1), while the β2 integrins, Mac-1 and LFA-1, bind to intercellular adhesion molecule-1 (ICAM-1) and ICAM-2, which are expressed on activated endothelium (Springer, 1990; Smith et al., 1990).

Previous findings have suggested that leukocyte-endothelial interaction plays an important role in the pathogenesis of splanchnic artery occlusion shock: indeed the E-selectin mediated step and ICAM-1 dependent leukocyte 'strong adhesion' have been shown to take place in this model of experimental shock (Altavilla et al., 1995a,b; Squadrito et al., 1994a).

In the present study, we investigated the leukocyte β1 integrin, VLA-4, and the endothelial molecule, VCAM-1, in experimental animals subjected to splanchnic artery occlusion shock. Our results suggest that VCAM-1/VLA-4 interaction plays an important role in the pathogenesis of experimental circulatory shock.

2. Materials and methods

2.1. Surgical procedures

Male Sprague-Dawley rats weighing 250–300 g were allowed access to food and water ad libitum. The rats were anaesthetized with urethane (1.3 g/kg, i.p.). After anaesthesia catheters were placed in the carotid artery and jugular vein. Blood pressure was monitored continuously using a Statham pressure transducer. After midline laparotomy, the celiac and superior mesenteric arteries were isolated near their aortic origins. During this procedure, the intestinal tract was maintained at 37°C by placing it between gauze pads soaked with warmed 0.9% NaCl solution.

Rats were given heparin (1000 U/kg, i.v.) and were observed for a 30-min stabilization period prior to either splanchnic ischaemia or sham ischaemia. Splanchnic artery occlusion shock was induced by clamping both the superior mesenteric artery and the celiac trunk resulting in total occlusion of these arteries for 45 min. After this period of occlusion the clamps were removed. Following reperfusion the rats were observed for 240 min. Sham shocked rats were subjected to the same surgical procedures as splanchnic artery occlusion shocked rats except that the arteries were not occluded.

2.2. Survival evaluation

Three hours before the splanchnic artery occlusion shock procedures, the treated rats received intravenously either anti-rat VCAM-1 (2 mg/kg) or anti-rat VLA-4 monoclonal antibodies (2 mg/kg) dissolved in 0.3 ml of a phosphate-buffered solution at pH 7.4 and control rats received a non-immune serum. Survival was evaluated for 4 h. In some experiments splanchnic artery occlusion shocked rats were treated with cloricromene (2 mg/kg) and control rats received the drug vehicle (1 ml/kg of a 0.9% NaCl solution).

2.3. Biological assay for tumour necrosis factor-α activity

Killing of L929 mouse tumour cells was used to measure TNF-α levels in serum on the basis of a standard assay (Ruff and Gifford, 1980). L929 cells in RPMI 1640 medium containing 5% fetal calf serum were seeded at 3×10^4 cells per well in 96-well microdilution plates and incubated overnight at 37°C in an atmosphere of 5% CO_2 in air. Serial dilutions of serum were made in a medium containing 1.0 μg of actinomycin D per ml and 100 μl of each dilution was added to the wells. On the next day, cell survival was assessed by fixing and staining the cells with crystal violet (0.2% methanol) and 0.1 ml of 1% sodium dodecyl sulphate was added to each well to solubilize the stained cells. The absorbance of each well was read at 490 nm with a model BT-100 Microelisa Autoreader. The percentage of cytotoxicity was calculated as $[1 - (A_{490}$ of sample$/A_{490}$ of control$)] \times 100$. One unit of TNF-α was defined as the amount giving 50% cell cytotoxicity. The TNF-α content of the sample was calculated by comparison with a calibration curve obtained with recombinant murine TNF-α (Nuclear Laser Medicine, Milan, Italy). To verify if the cytotoxicity tested was due to the presence of TNF-α or to other factors, we preincubated our samples for 2 h at 37°C with an excess of rabbit antirecombinant murine TNF-α polyclonal antibodies (Nuclear Laser Medicine, Milan, Italy) or with control rabbit serum. Our results showed that cytotoxicity against L929 cells was completely neutralized by rabbit antirecombinant TNF-α polyclonal antibodies but not by control rabbit serum.

2.4. Leukocyte count

Tail vein blood samples for leukocyte counting (Squadrito et al., 1992b) were taken at different time intervals (0 and 45 min before occlusion, and 80 min after reperfusion). The number of leukocytes (WBC $\times$ $10^3/mm^3$) is shown as the mean ± S.D.

2.5. Isolated aortic rings

The animals were killed 80 min after the start of reperfusion. Thoracic aortas were removed and placed in cold Krebs' solution of the following composition (mM): NaCl 118.4, KCl 4.7, $MgSO_4$ 1.2, $CaCl_2$ 2.5, KH_2PO_4 1.2, $NaHCO_3$ 25.0 and glucose 11.7. Then the aortas were cleaned of adherent connective and fat tissue and cut into rings approximately 2 mm in length. Rings were then placed under 1 g tension in an organ bath containing 10 ml

of Krebs' solution at 37°C and bubbled with 95% O_2 and 5% CO_2 (pH 7.4). All experiments were carried out in the presence of indomethacin (10 μM) in order to exclude the involvement of prostaglandins and their metabolites. Developed tension was measured with an isometric force transducer and recorded on a polygraph (Ugo Basile, Varese, Italy). After an equilibration period of 60 min during which time the rings were washed with fresh Krebs' solution at 15–20 min intervals and the basal tension was readjusted to 1 g, the tissue was exposed to phenylephrine (100 nM). When the contraction was stable, the functional integrity of endothelium was assessed from a relaxant response to acetylcholine (100 nM). The tissues were then washed occasionally for 30 min.

Endothelium-dependent relaxation was evaluated with cumulative concentrations of acetylcholine (10 nM–1 μM) in aortic rings precontracted with phenylephrine (100 nM). Relaxation of the rings was calculated as percent decrease of contractile force.

2.6. Immunohistochemistry

VCAM-1 expression was studied in thoracic aortas collected 80 min following the release of occlusion. Immunohistochemical evaluation was accomplished by staining 5-μm-thick cryostat sections according to the avidin-biotin-peroxidase complex procedure (Hsu et al., 1981). An average of two sections per immunohistochemical stain were cut from each sample, air-dried for 30 min and then fixed in cold acetone for 10 min. Endogenous peroxidases were blocked with horse serum for 15 min at room temperature prior to incubation with primary antibodies. Monoclonal antibodies consisted of anti-mouse VCAM-1 (clone: 429 MVCAM.A; isotype rat IgG2a, k) and were obtained from Pharmigen (San Diego, CA, USA). Biotinylated, species-specific second layer reagents were then applied, followed by avidin-biotin-horseradish peroxidase complex as a chromogenic substrate, as previously reported (Hsu et al., 1981). Sections were sampled to include the outermost 1 mm of tissue in direct contact with culture medium to ensure maximum exposure of tissue to mediators.

2.7. Cell flow cytometric analysis

The expression of the rat leukocyte cell surface molecule VLA-4 (CD49d) was investigated using a flow cytometric analysis with direct immunofluorescence: 100 μl of blood was incubated with 10 μl of monoclonal anti-rat VLA-4 antibodies conjugated with fluorescein isothiocyanate in a cold bath for 30 min.

The red cells were lysed by incubation of the samples for 20 min with a 0.86% ammonium chloride solution. The suspension obtained was washed with PBS and the cells were then put in PBS.

The percentage of cells expressing the VLA-4 receptor was calculated by cell flow cytometric analysis (Facsscan, Becton Dickinson, USA) using different 'gates' for the different subsets of leukocytes.

2.8. Lymphocyte binding to aortic endothelium

Autologous lymphocytes were isolated from the rats and were labeled according to a previously described method (Preston et al., 1993). These labeled lymphocytes were activated with concanavalin-A (10 μg/100 μl) and then placed in contact with the endothelial surface of a previously prepared aortic artery segment collected from untreated splanchnic artery occlusion shocked rats. These segments were treated either with an antibody anti-VCAM-1 at a dilution of 1:500 or with a non-immune serum (10 μl). Adherent lymphocytes were counted and the counts were averaged for 5 high-power fields at ×100 magnification using a microscope with epifluorescent illumination.

2.9. Drugs

Acetylcholine chloride, phenylephrine hydrochloride and indomethacin were obtained from Sigma. Monoclonal anti-rat mouse VCAM-1 antibodies (clone 429 MVCAM; isotype rat immunoglobulin G2a, k) were purchased from Pharmigen (San Diego, CA, USA). This antibody reacts with the VCAM-1 expressed on cytokine-stimulated vascular endothelium. Monoclonal anti-rat VLA-4 antibodies (clone MRα4; isotype: IgG2β, k) were also obtained from Pharmigen (San Diego, CA, USA). This antibody reacts with the α_4 subunit of VLA-4 ($\alpha_4\beta_1$ integrin). Control rats were injected with an immune mouse serum dissolved in the carrier vehicle (0.3 ml of phosphate-buffered saline solution at pH 7.4). Testing for the presence of endotoxin (chromogenic *Limulus amoebocyte* lysate assay) revealed < 1 pg/ml endotoxin in both immune and non-immune serum.

2.10. Statistical analysis

The significance of the difference between the means of groups was evaluated with analysis of variance (ANOVA) followed by Bonferroni's test and was considered significant when $P < 0.05$.

3. Results

3.1. Survival

Table 1 summarizes survival rate, percentage survival and survival time for the groups of rats subjected to splanchnic artery occlusion shock or sham shock. All sham rats survived the entire 4 h observation period. In contrast, in rats treated with vehicle, splanchnic artery occlusion shock produced a profound shock state characterized by a

Table 1
Effect of anti-VCAM-1 antibody (Ab), anti-VLA-4 antibody or non-immune serum on survival rate, percentage survival and survival time in splanchnic artery occlusion (SAO) shocked rats

Treatment	Hours after SAO				Survival time (min)
	2		4		
	Surviving	%	Surviving	%	
Sham + non-immune serum	10/10	100	10/10	100	> 240
Sham + Ab anti-VCAM-1 (2 mg/kg, i.v.)	10/10	100	10/10	100	> 240
Sham + Ab anti-VLA-4 (2 mg/kg, i.v)	10/10	100	10/10	100	> 240
SAO + non-immune serum	0/10	0	0/10	0	76 ± 10
SAO + Ab anti-VCAM-1 (2 mg/kg, i.v.)	10/10 [a]	100 [a]	9/10 [a]	90 [a]	227 ± 9 [a]
SAO + Ab anti-VLA-4 (2 mg/kg, i.v.)	10/10 [a]	100 [a]	8/10 [a]	80 [a]	205 ± 5 [a]

Animals received Ab anti-VCAM-1 (2 mg/kg), Ab anti-VLA-4 (2 mg/kg) or non-immune serum as an intravenous bolus 3 h before inducing SAO shock.
[a] $P < 0.001$ vs. SAO + vehicle.

high lethality: no rat survived at 2 h (survival time 76 ± 10 min). A passive immunization with specific antibodies against either VCAM-1 or VLA-4 significantly increased survival rate and time in shocked rats when compared with rats treated with vehicle (Table 1).

3.2. Serum TNF-α

Serum levels of TNF-α were undetectable in sham shocked rats treated with immune or non-immune serum. TNF-α was significantly increased in the serum collected from splanchnic artery occlusion shocked rats at the end of the reperfusion period (Table 2). Treatment with both anti-VCAM-1 antibody or anti-VLA-4 antibody did not change serum levels of the cytokine in splanchnic artery occlusion shocked rats (results not shown). The administration of cloricromene, an inhibitor of TNF-α biosynthesis, significantly blunted the serum levels of this cytokine (Table 2).

3.3. Monocyte and lymphocyte count

The administration of either the immune or non-immune serum did not modify monocyte and lymphocyte count in sham-shocked rats (Table 3). In contrast, splanchnic artery occlusion shock produced a marked decrease in both monocyte and lymphocyte count. Our data show that leukocyte count was markedly decreased at the end (80 min) of reperfusion (Table 3). The administration of either monoclonal anti-VCAM-1 antibody or monoclonal anti-VLA-4 antibody significantly ameliorated the reduction in both monocyte and lymphocyte count (Table 3).

Table 2
Serum TNF-α in splanchnic artery occlusion (SAO) shocked rats

Treatment	Serum TNF-α (U/ml)
Sham + vehicle	N.D.
Sham + cloricromene	N.D.
SAO + vehicle	328 ± 11
SAO + cloricromene	10 ± 2 [a]

Serum was collected 80 min after clamp removal. Each point represents the mean ± S.D. from six experiments. Animals received cloricromene (2 mg/kg) or vehicle (1 ml/kg of a 0.9% NaCl solution) as an intravenous bolus 5 min before inducing SAO shock. [a] $P < 0.001$ vs. SAO + vehicle. N.D. = not detectable.

3.4. Relaxant response to acetylcholine

Addition of phenylephrine (100 nM) to the organ bath contracted intact aortic rings (80–90% of the maximum

Table 3
Monocyte and lymphocyte count in splanchnic artery occlusion (SAO) shocked rats

Treatment	Monocyte count × 10^3/mm^3		
	Basal	Occlusion	Reperfusion
Sham + non-immune serum	0.82 ± 0.09	0.93 ± 0.02	0.92 ± 0.02
Sham + Ab anti-VCAM-1 (2 mg/kg, i.v.)	0.93 ± 0.03	0.95 ± 0.04	0.89 ± 0.02
Sham + Ab anti-VLA-4 (2 mg/kg, i.v.)	0.90 ± 0.06	0.94 ± 0.07	0.93 ± 0.05
SAO + non-immune serum	0.91 ± 0.04	0.92 ± 0.01	0.59 ± 0.01 [a]
SAO + Ab anti-VCAM-1 (2 mg/kg, i.v.)	0.90 ± 0.03	0.87 ± 0.05	0.82 ± 0.05 [b]
SAO + Ab anti-VLA-4 (2 mg/kg, i.v.)	0.82 ± 0.08	0.80 ± 0.06	0.96 ± 0.09 [b]
	Lymphocyte count × 10^3/mm^3		
	Basal	Occlusion	Reperfusion
Sham + non-immune serum	14 ± 2	16 ± 4	15 ± 5
Sham + Ab anti-VCAM-1 (2 mg/kg, i.v.)	13 ± 5	14 ± 3	13 ± 4
Sham + Ab anti-VLA-4 (2 mg/kg, i.v.)	15 ± 3	13 ± 2	12 ± 4
SAO + non-immune serum	14 ± 3	14 ± 1	9.8 ± 1 [a]
SAO + Ab anti-VCAM-1 (2 mg/kg, i.v.)	15 ± 1	12 ± 3	11 ± 2 [b]
SAO + Ab anti-VLA-4 (2 mg/kg, i.v.)	13 ± 4	11 ± 2	11 ± 3 [b]

Animals received Ab anti-VCAM-1 (2 mg/kg), Ab anti-VLA-4 (2 mg/kg) or a non-immune serum as an i.v. bolus 3 h before SAO shock. [a] $P < 0.05$ vs. sham + vehicle; [b] $P < 0.05$ vs. SAO vehicle. Each point represents the mean ± S.D. of six experiments.

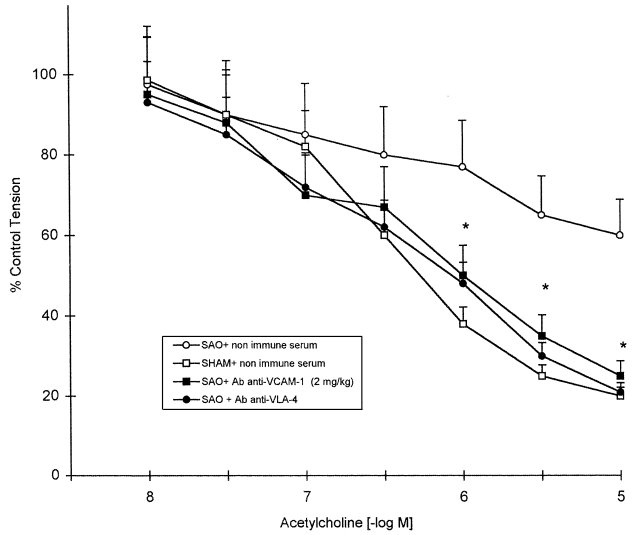

Fig. 1. Relaxant effect of acetylcholine in aortic rings (contracted with phenylephrine, 100 nM) of sham shocked rats and splanchnic artery occlusion (SAO) shocked rats treated either with non-immune serum, anti-VCAM-1 antibody (2 mg/kg, i.v., 3 h before SAO), anti-VLA-4 antibody (2 mg/kg, i.v). Each point represents the mean ± S.D. from seven experiments. * $P < 0.01$ vs. SAO + non-immune serum.

response). These rings were relaxed in a concentration-dependent manner by acetylcholine (10 nM–10 μM). The relaxant effect of acetylcholine in aortic rings from shocked rats was significantly lower in splanchnic artery occlusion shocked rats than in sham shocked rats (Fig. 1). Administration of monoclonal antibody raised either against VCAM-1 or VLA-4 significantly improved responsiveness to acetylcholine (Fig. 1).

3.5. VCAM-1 expression on aortic endothelium

VCAM-1 expression was studied in thoracic aortae collected 80 min following the release of occlusion. Immunohistochemical evaluation indicated that a very low constitutive expression of VCAM-1 was present in sham shocked rats (Table 4). In contrast, samples of thoracic aortae obtained from splanchnic artery occlusion shocked rats had an increase in VCAM-1 staining (Table 4). Aortic endothelium obtained from splanchnic artery occlusion shocked rats treated with cloricromene showed a marked decrease in the endothelium expression of VCAM-1 (Table 4).

Table 4
VCAM-1 expression in aortic rings from splanchnic artery occlusion (SAO) or sham shocked rats

Treatment	Immunohistochemical staining for VCAM-1
Sham + vehicle	− (9)
Sham + cloricromene	− (6)
SAO + vehicle	+ + (9) + (4)
SAO + cloricromene	− (8) + (4)

Thoracic aortae were collected 80 min after clamp removal. The number of experiments is between parentheses. Rats received cloricromene (2 mg/kg) or vehicle (1 ml/kg of a 0.9% NaCl solution) as an intravenous bolus 5 min before splanchnic artery occlusion shock was induced. Very low presence of VCAM-1 (−); mild expression of VCAM-1 (+); marked increase in the expression of VCAM-1 (+ +).

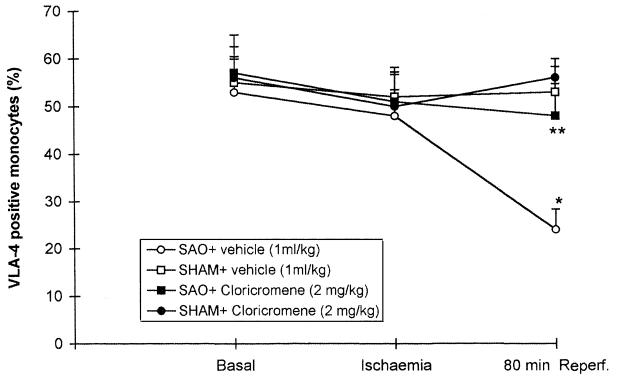

Fig. 2. VLA-4 positive monocytes in sham shocked rats and splanchnic artery occlusion (SAO) shocked rats treated either with vehicle (1 ml/kg, i.v.) or cloricromene (2 mg/kg, i.v., 5 min before SAO). Each point represents the mean ± S.D. from seven experiments. * $P < 0.01$ vs. SAO + vehicle; * * $P < 0.001$ vs. sham + vehicle.

3.6. Leukocyte VLA-4

The percentage of leukocytes expressing the VLA-4 receptor was studied by means of cytometric flow analysis. In sham shocked rats the subsets of leukocytes bearing the VLA-4 receptor were monocytes (56 ± 7%) and lymphocytes (35 ± 8%) while neutrophils were not significantly positive for the VLA-4 receptor (< 1%). The kinetics of VLA-4 positive leukocytes was determined in rats at different times: 0 and 45 min after occlusion and 80 min post-reperfusion. The percentage of both monocytic and lymphocytic positive cells was significantly reduced at the end of reperfusion (Fig. 2 and Fig. 3). The administration of cloricromene significantly increased the number of VLA-4 positive leukocytes (Fig. 2 and Fig. 3).

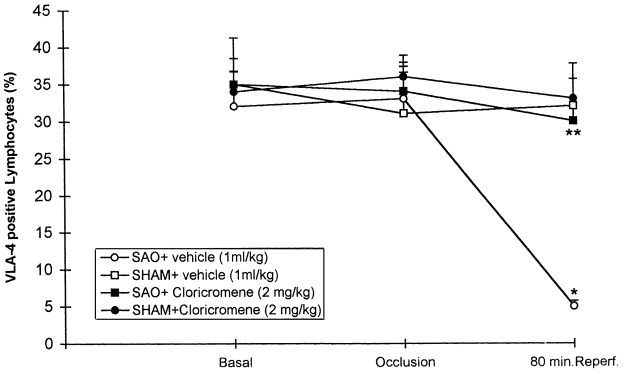

Fig. 3. VLA-4 positive lymphocytes in sham shocked rats and splanchnic artery occlusion (SAO) shocked rats treated either with vehicle (1 ml/kg, i.v.) or cloricromene (2 mg/kg, i.v., 5 min before SAO). Each point represents the mean ± S.D. from seven experiments. * $P < 0.01$ vs. SAO + vehicle; * * $P < 0.001$ vs. sham + vehicle.

Table 5
Effects of anti-VCAM-1 antibody on lymphocyte adherence to aortic segments from splanchnic artery occlusion shocked rats

Treatment	Lymphocyte adherence (%)
Lymphocytes alone	20 ± 5
Lymphocytes + concanavalin-A	78 ± 8 [a]
Lymphocytes + concanavalin-A + non-immune serum	82 ± 4 [a]
Lymphocytes + concanavalin-A + Ab anti-VCAM-1	28 ± 3 [b]

Thoracic aortae were collected 80 min after clamp removal. Lymphocytes were activated with concanavalin-A (10 μg/100 μl) and then placed in contact with the endothelial surface of previously prepared aortic segments pretreated with an anti-VCAM-1 antibody used at a dilution of 1:500 or with a non-immune serum (10 μl). Each point represents the mean ± S.D. of seven segments. [a] $P < 0.01$ vs. lymphocytes alone; [b] $P < 0.001$ vs. lymphocytes + non-immune serum.

3.7. Lymphocytes vascular adherence

Activated lymphocytes adhered in a significant percentage to the isolated aortic segments from untreated splanchnic artery occlusion shocked rats (Table 5). Pretreatment with the anti-VCAM-1 antibody significantly prevented the adherence of lymphocytes to the aortic endothelium. (Table 5). The result of this experiment indicates that our antibody efficiently blocks the binding of lymphocytes to the rat endothelium and efficiently cross-reacts with rat VCAM-1.

4. Discussion

Splanchnic artery occlusion shock is an experimental type of circulatory shock which is the consequence of a prolonged ischaemia of the splanchnic region (Sturniolo et al., 1988). Splanchnic artery occlusion shock is characterized by impaired activity of the reticuloendothelial system (Sturniolo et al., 1989), elevated macrophage and plasma levels of thromboxane B_2 (Squadrito et al., 1992a), increased plasma levels of platelet activating factor (Squadrito et al., 1991a; Zingarelli et al., 1992) and enhanced macrophage and serum levels of tumour necrosis factor (TNF-α) (Squadrito et al., 1992b). Furthermore, splanchnic artery occlusion shock produces an irreversible circulatory failure which leads to the death of the animals within 75–90 min after the release of the occlusion (Squadrito et al., 1989, 1994b).

Previous findings from our laboratory have shown that endothelium-leukocyte interaction plays an important role in the pathogenesis of splanchnic artery occlusion shock: indeed we have shown that the mechanisms underlying the leukocyte rolling and accumulation on the endothelium (mechanisms which are mediated by the endothelial adhesion molecules E-selectin and ICAM-1) are operative in this model of experimental shock.

However, the E-selectin and ICAM-1 mediated phenomena do not allow us to precisely identify the importance of the several subsets of leukocytes in experimental shock: in fact E-selectin interacts with granulocyte and lymphocyte subsets, while ICAM-1 binds to all types of leukocytes (Gearing and Newman, 1993). Indeed our previous work has pointed out the key importance of neutrophils in the pathogenesis of splanchnic ischaemia-reperfusion injury. In contrast with these previous findings, less is known about the adhesion molecule selectively specialized for the recruitment of lymphocytes and monocytes in splanchnic artery occlusion shock.

The integrin VLA-4 mediates lymphocyte and monocyte binding to the endothelial adhesion receptor vascular cell adhesion molecule-1 (VCAM-1). This endothelial adhesion molecule, which belongs to the immunoglobulin superfamily, has a very low constitutive presence in the resting endothelium, but its expression, like that of ICAM-1, is highly induced on activated endothelium by proinflammatory cytokines such as TNF-α (Adams and Shaw, 1994).

Therefore the aim of our work was to investigate in our model of shock the involvement of the VCAM-1 mediated binding of lymphocytes and monocytes to the endothelium.

Our data showed that a passive immunization with a specific antibody raised against either VCAM-1 or VLA-4 was able to increase the survival of rats subjected to the splanchnic artery occlusion shock procedures: indeed this finding clearly indicates the key role played by the VLA-4/VCAM-1 interaction in the pathogenesis of this type of experimental shock.

The endothelium of sham shocked rats had a very low staining for the endothelial receptor VCAM-1: this finding is in close agreement with previous findings showing the presence of a very low constitutive expression of this adhesion molecule in non-stimulated tissues (Koizumi et al., 1992). In contrast, in rats subjected to splanchnic ischaemia-reperfusion injury we found an increased presence of VCAM-1. Since our shocked rats had increased serum levels of TNF-α, it might be hypothesized that this inflammatory cytokine is responsible for over-expression of this endothelial adhesion receptor in this in vivo model of shock. As a matter of fact, inhibition of TNF-α reduced the expression of VCAM-1. Indeed, this latter finding confirms previous in vitro experiments showing that VCAM-1 expression can be up-regulated by proinflammatory cytokines (Bevilacqua, 1993). However, in vitro VCAM-1 expression requires at least 3 h following stimulation with inflammatory cytokines such as TNF-α. In contrast we found that in our in vivo model VCAM-1 is up-regulated within 80 min following the release of occlusion. It could be hypothesized that, under our experimental conditions, the inflammatory stimuli that prime the expression of VCAM-1 are stronger than in vitro. Indeed, other inflammary cytokines, including interleukin-1 and interleukin-6, have been shown to be increased in the bloodstream of rats subjected to splanchnic artery occlusion

shock. Furthermore, we have previously shown that inhibition of TNF-α protects against splanchnic artery occlusion shock, thus confirming the pivotal role played by this cytokine in this type of circulatory shock (Squadrito et al., 1991b).

Lymphocytes and monocytes in sham or splanchnic artery occlusion shocked rats were the only two leukocyte subsets that expressed in a significant percentage the VLA-4 receptors: this finding is in agreement with previous data (Adams and Shaw, 1994). Indeed, it has been proposed that neutrophils may also bear the VLA-4 receptor (Kubes et al., 1995). However, at least in our experimental conditions, neutrophils did not express the integrin VLA-4. Surprisingly, we observed that the percentage of VLA-4 positive lymphocytes and monocytes decreased markedly at the end of reperfusion in splanchnic artery occlusion shocked rats. Furthermore, the total number of both subsets of leukocytes was also reduced at the end of reperfusion. The reduction in the percentage of VLA-4 positive monocytes and lymphocytes paralleled in terms of absolute cell count number the decrease in the total number of monocytes and lymphocytes. In other words, the 50% decrease in VLA-4 positive monocytes and the 75% decrease in VLA-4 positive lymphocytes corresponded roughly with the reduction of 0.3 cells $\times 10^3$ per mm^3 and of 4 cells $\times 10^3$ per mm^3 found in the absolute count of monocytes and lymphocytes, respectively.

The administration of cloricromene, a previously described inhibitor of TNF-α synthesis (Squadrito et al., 1991b, 1992c), reduced the expression of VCAM-1 on aortic endothelium and increased the percentage of circulating VLA-4 positive leukocytes.

All these findings, taken together, suggest that during reperfusion the up-regulation of VCAM-1 on endothelium causes VLA-4 positive leukocytes to bind to the vascular endothelium, and in turn to disappear from the circulation. This mechanism explains why we measured a decreased percentage of leukocytes bearing the VLA-4 receptor in rats subjected to splanchnic artery occlusion shock. In agreement with this hypothesis passive immunization with specific antibodies against either VCAM-1 or VLA-4 reversed the decrease in monocyte and lymphocyte count.

Furthermore, since inhibition of TNF-α reduced the expression of VCAM-1 and increased the percentage of VLA-4 positive leukocytes, it might be speculated that in circulatory shock this inflammatory cytokine causes the VLA-4/VCAM-1 interaction. In addition, the VLA-4 positive leukocytes may also extravasate and accumulate in peripheral organs such as the ileum and the lungs. Our present data showed that aortic rings from splanchnic artery occlusion shocked rats had a markedly reduced responsiveness to the vasorelaxant effects of acetylcholine, suggesting that the NO produced by the endothelial constitutive NO synthase is reduced in splanchnic artery occlusion shocked rats, as previously reported (Squadrito et al., 1994a). Indeed, our present data also showed that the administration of a specific antibody raised against VCAM-1, probably by reducing monocyte and lymphocyte binding to the endothelium, rectified the impairment in endothelial NO production. This experimental evidence led us to hypothesize that all these subsets of leukocytes may contribute to the development of endothelial dysfunction in circulatory shock.

In conclusion, our findings suggest that VCAM-1/VLA-4 interaction plays an important role in the pathogenesis of experimental circulatory shock.

Acknowledgements

This work was supported by Ministero Pubblica Istruzione, Fondi 40% and 60%, and in part by a grant from CNR Italy (93.04296.CT04). We gratefully acknowledge the skilful technical assistance of Carole Campbell in the preparation of the manuscript.

References

Adams, D.H. and S. Shaw, 1994, Leukocyte-endothelial interactions and regulation of leukocyte migration, Lancet 343, 831.

Altavilla, D., F. Squadrito, P. Canale, M.P. Ioculano, G.M. Campo, G. Squadrito, L. Ammendolia and A.P. Caputi, 1995a, E-selectin involvement in the pathogenesis of splanchnic artery occlusion shock, Eur. J. Pharmacol. 272, 223.

Altavilla, D., F. Squadrito, P. Canale, M.P. Ioculano, G.M. Campo, G. Squadrito, A. Saitta and A.P. Caputi, 1995b, Tumor necrosis factor induces E-selectin production in splanchnic artery occlusion shock, Am. J. Physiol. 268, H1412.

Bevilacqua, M.P., 1993, Endothelial-leukocyte adhesion molecules, Annu. Rev. Immunol. 11, 767.

Butcher, E.C., 1991, Leukocyte-endothelial cell recognition: three (or more) steps to specificity and diversity, Cell 67, 1033.

Gearing, A.J.H. and H. Newman, 1993, Circulating adhesion molecules in disease, Immunol. Today 14, 506.

Hsu, S.M., L. Raine and H. Fanger, 1981, A comparative study of the peroxidase-antiperoxidase method and an avidin-biotin complex method for studying polypeptide hormones with radioimmunoassay antibodies, Am. J. Clin. Pathol. 75, 734.

Hubere, A.R., S.L. Kunkel, R.F. Todd III and S.J. Weiss, 1991, Regulation of transendothelial neutrophil migration by endogenous interleukin-8, Science 254, 99.

Koizumi, M., N. King, R. Lobb, C. Benjamin and D.K. Podolski, 1992, Expression of vascular adhesion molecules in inflammatory bowel disease, Gastroenterology 103, 840.

Kubes, P., X.F. Niu, C.W. Smith, M.E. Kehrli, P.H. Reinhardt and R.C. Woodman, 1995, A novel beta (1)-dependent adhesion pathway on neutrophils: a mechanism invoked by dihydrocytochalasin B on endothelial transmigration, FASEB J. 11, 1103.

Lawrence, M.B. and T.A. Springer, 1991, Leukocytes roll on a selectin at physiologic flow rates: distinction from and prerequisite for adhesion through integrins, Cell 65, 859.

Preston, P.M., C.G.D. Brown, G. Entrican, W. Richardson and R. Boid, 1993, Synthesis of tumour necrosis factor-α and interferons by mononuclear cells from *Theileria annulata*-infected cattle, Parasite Immunol. 15, 525.

Rot, A., 1992, Endothelial cell binding of NAP-1/IL-8: role in neutrophil emigration, Immunol. Today 13, 291.

Ruff, M.R. and G.E. Gifford, 1980, Purification and physicochemical characterization of rabbit tumor necrosis factor, J. Immunol. 25, 1671.

Schall, T.J., 1991, Biology of the RANTES/SIS cytokine family, Cytokine 3, 165.

Shimizu, Y., W. Newman, Y. Tanaka and S. Shaw, 1992, Lymphocyte interactions with endothelial cells, Immunol. Today 13, 1112.

Smith, C.W., S.D. Marlin, R. Rothlein, M.B. Lawrence, L.V. Mcintire and D.C. Anderson, 1990, Role of ICAM-1 in the adherence of human neutrophils to human endothelial cells in vitro, in: Leukocyte Adhesion Molecules. Structure, Function, and Regulation, eds. T.A. Springer, D.C. Anderson, A.S. Rosenthal and R. Rothlein (Springer-Verlag, New York, NY) p. 170.

Smith, C.W., T.K. Kishimoto, O. Abbassi et al., 1991, Chemotactic factors regulate lectin adhesion molecule 1 (LECAM-1)-dependent neutrophil adhesion to cytokine stimulated endothelial cells in vitro, J. Clin. Invest. 87, 609.

Springer, T.A., 1990, Adhesion receptors of the immune system, Nature 346, 425.

Squadrito, F., R. Sturniolo, D. Altavilla, G.M. Campo, G.R. Trimarchi, R. Scuri and A.P. Caputi, 1989, Effects of fructose 1,6 diphosphate on splanchnic artery occlusion shock in the rat, Resuscitation 18, 299.

Squadrito, F., R. Sturniolo, D. Altavilla, G. Santoro, G. Campo, A. Arena and A.P. Caputi, 1991a, Platelet activating factor involvement in splanchnic artery occlusion shock in rats, Eur. J. Pharmacol. 192, 47.

Squadrito, F., M. Prosdocimi, D. Altavilla, B. Zingarelli and A.P. Caputi, 1991b, Cloricromene, Cardiovasc. Drug Rev. 9, 357.

Squadrito, F., D. Altavilla, B. Zingarelli, M. Ioculano, G.M. Campo, A. Saitta, G. Urna, G. Spignoli and A.P. Caputi, 1992a, Protective effects of G 619, a dual thromboxane synthase inhibitor and thromboxane A_2 receptor antagonist, in splanchnic artery occlusion shock, J. Cardiovasc. Pharmacol. 19, 115.

Squadrito, F., D. Altavilla, M. Ioculano, G. Calapai, B. Zingarelli, A. Saitta, G.M. Campo, A. Rizzo and A.P. Caputi, 1992b, Passive immunization with antibodies against tumor necrosis factor (TNF-α) protects from the lethality of splanchnic artery occlusion shock, Circ. Shock 37, 236.

Squadrito, F., D. Altavilla, G.M. Campo, G. Calapai, M. Ioculano, B. Zingarelli, A. Saitta, M. Prosdocimi and A.P. Caputi, 1992c, Cloricromene, a coumarine derivative, protects against lethal endotoxin shock in rats, Eur. J. Pharmacol. 210, 107.

Squadrito, F., D. Altavilla, P. Canale, M.P. Ioculano, G.M. Campo, L. Ammendolia, G. Squadrito, A. Saitta, G. Calapai and A.P. Caputi, 1994a, Contribution of intercellular adhesion molecule 1 (ICAM-1) to the pathogenesis of splanchnic artery occlusion shock in the rat, Br. J. Pharmacol. 113, 912.

Squadrito, F., D. Altavilla, P. Canale, M. Ioculano, G.M. Campo, L. Ammendolia, M. Ferlito, B. Zingarelli, G. Squadrito, A. Saitta and A.P. Caputi, 1994b, Participation of tumor necrosis factor and nitric oxide in the mediation of vascular dysfunction in splanchnic artery occlusion shock, Br. J. Pharmacol. 113, 1153.

Sturniolo, R., D. Altavilla, M.C. Berlinghieri, F. Squadrito and A.P. Caputi, 1988, Splanchnic artery occlusion shock in the rat: effects of calcium entry blockers nimodipine and verapamil, Circ. Shock 24, 43.

Sturniolo, R., F. Squadrito, D. Altavilla, G.R. Trimarchi, M. Prosdocimi and A.P. Caputi, 1989, Cloricromene improves survival rate and macrophage function in splanchnic artery occlusion shock in rats, Circ. Shock 28, 267.

Taub, D.D., K. Conlom, A.R. Lloyd, J.J. Oppenheim and D.J. Kelvin, 1993, Preferential migration of activated human $CD4^+$ and $CD8^+$ T cells in response to MIP-1α and MIP-1β, Science 260, 355.

Zimmerman, G.A., S.M. Prescott and T.M. Mcintyre, 1992, Endothelial cell interactions with granulocytes: tethering and signaling molecules, Immunol. Today 13, 93.

Zingarelli, B., F. Squadrito, M. Ioculano, D. Altavilla, F. Bussolino, G.M. Campo and A.P. Caputi, 1992, Platelet activating factor interaction with tumor necrosis factor and myocardial depressant factor in splanchnic artery occlusion shock, Eur. J. Pharmacol. 222, 13.