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E-selectin involvement in the pathogenesis of splanchnic artery occlusion shock

Domenica Altavilla [a,*], Francesco Squadrito [a], Patrizia Canale [a], MariaPatrizia Ioculano [a], Giuseppe M. Campo [a], Giovanni Squadrito [b], Letteria Ammendolia [a], Achille P. Caputi [a]

[a] *Institute of Pharmacology, School of Medicine, Piazza XX Settembre 4, 98122 Messina, Italy*
[b] *Department of Internal Medicine, University of Messina, Italy*

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Abstract

We investigated the involvement of E-selectin 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 tumor necrosis factor-α, white blood cell count, mean arterial blood pressure and myeloperoxidase activity were determined. Splanchnic artery occlusion-shocked rats had a decreased survival time (85 ± 8 min, while sham-shocked rats survived more than 4 h), reduced mean arterial blood pressure, increased serum levels of tumor necrosis factor-α (186 ± 9 U/ml) and myeloperoxidase activity in the ileum (0.10 ± 0.04 U $\times 10^{-3}$/g tissue) and in the lung (1.5 ± 0.06 U $\times 10^{-3}$/g tissue). Shocked rats showed histological alterations in the ileum and in the lung. Administration of a hyperimmune serum containing specific antibodies raised against E-selectin significantly increased survival time (225 ± 10 min), reduced leukopenia and myeloperoxidase activity both in the ileum (0.035 ± 0.001 U $\times 10^{-3}$/g tissue) and in the lung (0.3 ± 0.005 U $\times 10^{-3}$/g tissue), improved the cardiovascular changes and reduced the histological alterations in the ileum and lung. Our data are consistent with an involvement of E-selectin in the pathogenesis of splanchnic artery occlusion shock.

Keywords: E-selectin; Splanchnic artery occlusion shock; Leukocyte accumulation

1. Introduction

Leukocyte adhesion to the endothelium is a process that plays a key role in the mechanisms underlying inflammation (Mackay and Imhof, 1993). This phenomenon represents the target for drugs that therapeutically modify the inflammatory response, since they mediate the initial attachment of leukocytes to the endothelium. Recent evidence has also suggested that E-selectin is involved in the mediation of myocardial reperfusion injury (Altavilla et al., 1994a), thus suggesting that this adhesion molecule may be also important in ischaemic and/or low-flow states.

Circulatory shock is a low-flow state in which leukocytes contribute to the full development of the syndrome. It has been suggested that leukocyte depletion, induced by administering vinblastine, is able to increase the resistance of rats to an experimental model of circulatory shock such as splanchnic artery occlusion shock (Canale et al., 1993).

Splanchnic artery occlusion shock is an experimental type of circulatory shock which is the consequence of a prolonged ischaemia of the splanchnic region (Squadrito et al., 1991). This model of shock is characterized by a marked decrease in systemic blood pressure and leukopenia (Sturniolo et al., 1988) as well as by disturbances in reticuloendothelial system activity (Sturniolo et al., 1989), increased macrophage and plasma levels of thromboxane B_2 (Squadrito et al., 1992a) and elevated plasma levels of platelet-activating factor (Zingarelli et al., 1992). It has also been suggested that tumor necrosis factor, besides its important role in the pathogenesis of Gram-negative septic shock (Tracey et al., 1989), may also represent an important mediator of splanchnic artery occlusion shock. In fact

* Corresponding author. Tel. 39 90 712533, fax 39 90 661029.

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enhanced macrophage and serum levels of tumor necrosis factor-α have been measured during this type of shock. Furthermore, a passive immunization with specific antibodies raised against this pleiotropic cytokine has been shown to be able to protect experimental animals from the lethality of splanchnic artery occlusion shock (Squadrito et al., 1992b).

Previous findings have shown that tumor necrosis factor-α enhances the adherence of leukocytes to the vascular endothelium (Pober and Cotran, 1990). This phenomenon is mediated, at least in part, by E-selectin and ICAM-1 (intercellular adhesion molecule) which are induced in vitro by the addition of recombinant tumor necrosis factor-α (Mantovani and Dejana, 1989). In light of these observations we investigated in vivo the role played by E-selectin in the pathogenesis of splanchnic artery occlusion shock. A passive immunization with specific antibodies raised against E-selectin significantly reduced leukocyte accumulation and markedly protected rats from the lethality induced by splanchnic artery occlusion shock.

2. Materials and methods

2.1. Surgical procedures

Male Sprague-Dawley rats weighing 250–300 g were permitted 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 exposed 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 all 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, treated rats received intravenously specific anti E-selectin antibodies (2 mg/kg) dissolved in 0.3 ml of a phosphate-buffered solution at pH 7.4 and control rats received the carrier vehicle. Survival was evaluated for 4 h.

2.3. Arterial blood pressure

Animals were anaesthetized with urethane (1.3 g/kg intraperitoneally) and a cannula (PE 50) was inserted into the left common carotid artery as described elsewhere (Caputi et al., 1980). The arterial catheter was connected to a pressure transducer. The pressure pulse triggered a cardiotachometer, and arterial blood pressure was displayed on a polygraph. Arterial blood pressure is reported as mean arterial pressure in mmHg. Rats were subjected to the same experimental protocol described above.

2.4. Biological assay for tumor necrosis factor-α activity

Killing of L929 mouse tumor cells was used to measure tumor necrosis factor-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 (drawn at different time intervals) were made in a medium containing 1.0 μg of actinomycin D per ml and 100 μl of each dilution were 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 $[1-(\text{Absorbance}_{490}\text{ of sample}/\text{A}_{490}\text{ of control})] \times 100$. One unit of tumor necrosis factor-α was defined as the amount giving 50% cell cytotoxicity. Tumor necrosis factor-α content in the sample was calculated by comparison with a calibration curve performed with recombinant murine tumor necrosis factor-α (Nuclear Laser Medicine, Italy). To verify if the cytotoxicity tested was due to the presence of tumor necrosis factor-α or to other factors, we preincubated our samples for 2 h at 37°C with an excess of rabbit antirecombinant murine tumor necrosis factor-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 anti-recombinant tumor necrosis factor-α polyclonal antibodies but not by control rabbit serum.

2.5. Myeloperoxidase activity

Leukocyte accumulation was investigated using the activity of myeloperoxidase. Myeloperoxidase activity

was determined in intestinal mucosa and in the left lung. The samples were obtained at 0 and 45 min before reperfusion (release of the arterial clamp) and at 80 min after reperfusion. The samples were first homogenized in a solution containing 20 mM of potassium phosphate buffer (pH 7.4), 0.01 M EDTA, 50 U/ml of a protease inhibitor (aprotinin) in proportions of 1:10 (w/v) and then centrifuged for 30 min at 20000 × *g* at 4°C. The supernatant of each sample was then discarded and the pellet was immediately frozen on dry ice. The pellet was frozen for 14 h before sonication. After the thawing, the resulting pellet was added to a buffer solution consisting of 0.5% hexacyltrimethylammonium bromide (Sigma Chemical, St. Louis, MO, USA) dissolved in 50 nM potassium phosphate buffer (pH 6) containing 30 U/ml of protease inhibitor. Each sample was then sonicated for 1 min at intensity 2 and at a temperature of 4°C. After the sonication the samples were allowed to chill on ice for approximately 30 min, and then they were centrifuged for 30 min at 40000 × g at 4°C. An aliquot of the supernatant was then allowed to react with 0.167 mg/ml *o*-dianisidine dihydrochloride (Sigma Chemical) and 0.0010% H_2O_2, and the rate of change in absorbance was measured at 405 nm in a microtitre plate reader. Myeloperoxidase activity was defined as the quantity of enzyme degrading 1 μmol of peroxide/min at 25°C and was expressed in milliunits per gram weight ($U \times 10^{-3}$) of tissue.

2.6. Histology

In a group of animals subjected to splanchnic artery occlusion shock, the ileum and the lung were removed at different time intervals (0 and 45 min before occlusion, and 80 min after reperfusion) and examined. Representative specimens were taken and fixed in 10% formalin and were later processed for histological examinations as elsewhere described (Squadrito et al., 1992b).

2.7. Leukocyte count

Tail vein blood samples for the leukocyte count (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 (white blood cells $\times 10^3/mm^3$) is reported as mean ± S.D.

2.8. Drug

Monoclonal mouse anti-human E-selectin antibodies (clone BBIG-E5, isotype: IgG_1) were purchased from British Biotechnology Products (Abingdon, UK). Control rats were administered with an isotype matched murine monoclonal antibody raised against human MHC antigen [HLA-(DR) Class II, clone B-F1 isotype IgG_1], obtained from Serotec (Oxford, UK). Preliminary experiments have shown that the anti-human E-selectin antibody stains rat endothelial cells and, in addition, this antibody was found to inhibit rat neutrophil adherence to rat endothelium 'in vitro'. Furthermore, the anti-human E-selectin antibody did not block either ICAM-1 or P-selectin. Finally, the anti-human E-selectin antibodies have been previously shown to block the E-selectin mediated effect in an experimental rat model of myocardial injury (Altavilla et al., 1994a). Control rats were injected with the same amount of a murine anti-human MHC antibody.

2.9. Statistical analysis

The difference between the means of two groups was evaluated with an ANOVA followed by Bonferroni's test and was considered significant at $P < 0.05$.

3. Results

3.1. Survival

Sham-shocked rats, treated either with anti MHC antibodies or with anti E-selectin antibodies, survived the entire 4-h observation period (Fig. 1). In contrast, in rats treated with anti MHC antibodies, splanchnic artery occlusion shock produced a profound shock state characterized by a high lethality and no rats survived at 2 h (survival time = 85 ± 8 min). A passive immuniza-

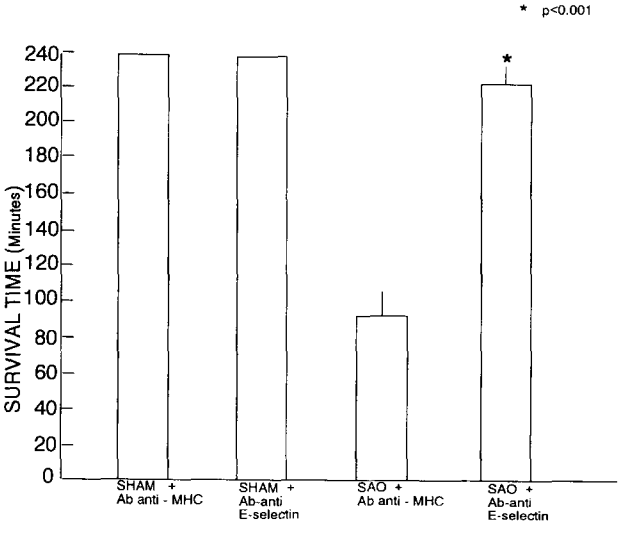

Fig. 1. Effects of a passive immunization with either anti MHC antibodies or specific antibodies against E-selectin on survival time in rats subjected to splanchnic artery occlusion shock (SAO). The antibodies (2 mg/kg, i.v.) were injected 3 h before splanchnic artery occlusion. Each point represents the mean ± S.D. of eight experiments. * $P < 0.001$ vs SAO + Ab anti MHC.

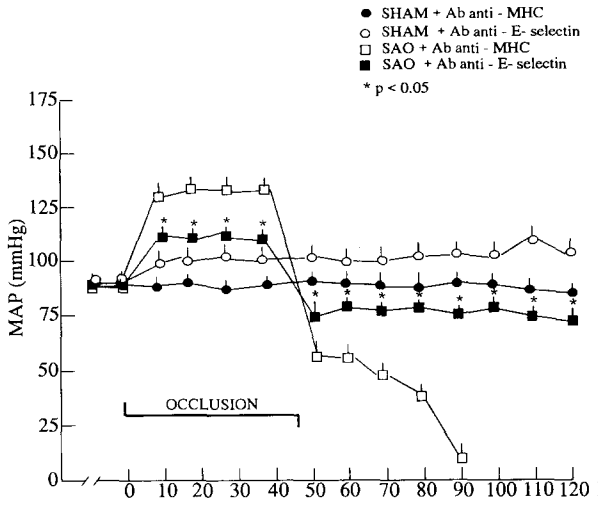

Fig. 2. Effects of anti E-selectin antibodies (2 mg/kg i.v.) or anti MHC antibodies (2 mg/kg i.v) on mean arterial blood pressure in rats subjected to splanchnic artery occlusion shock. Each point represents the mean ± S.D. of eight experiments. * $P < 0.05$ vs SAO + Ab anti MHC.

tion with specific antibodies raised against E-selectin significantly protected rats from the lethality induced by splanchnic artery occlusion shock: in fact survival time in these animals was 225 ± 10 min.

3.2. Arterial blood pressure

Occlusion of the splanchnic arteries produced a marked increase in mean arterial blood pressure. Subsequently pressure decreased upon release of the occlusion (Fig. 2). The administration of specific antibodies raised against E-selectin significantly blunted the reduction in mean arterial blood pressure (Fig. 2).

3.3. Serum tumor necrosis factor-α

Serum levels of tumor necrosis factor-α were undetectable in sham shocked rats treated either with anti MHC antibodies or anti E-selectin antibodies. Tumor necrosis factor-α was also undetectable during the occlusion period. In contrast serum tumor necrosis factor-α was significantly enhanced at the end of the reperfusion period in splanchnic artery occlusion-shocked rats (186 ± 9 U/ml). Treatment with anti E-selectin antibodies did not modify tumor necrosis factor-α levels during reperfusion (169 ± 9 U/ml).

3.4. Myeloperoxidase activity

The kinetics of ileal and pulmonary leukocyte infiltration in splanchnic artery occlusion shocked rats was determined by measuring the myeloperoxidase activity in rats at different times: 0 and 45 min after occlusion, and 80 min post reperfusion. Myeloperoxidase levels were significantly increased in the ileum (0.10 ± 0.04 $U \times 10^{-3}$/g tissue) and in the lung (1.5 ± 0.05 $U \times 10^{-3}$/g tissue) at 80 min after reperfusion (Figs. 3 and 4) in shocked rats pretreated with anti MHC antibodies.

A passive immunization with specific antibodies raised against E-selectin significantly lowered the increase in ileal (0.035 ± 0.001 $U \times 10^{-3}$/g tissue; Fig. 3) and pulmonary myeloperoxidase activity (0.3 ± 0.005 $U \times 10^{-3}$/g tissue; Fig. 4).

3.5. Leukocyte count

The administration of either anti MHC antibodies or anti E-selectin antibodies did not modify the leukocyte count in sham-shocked rats (Fig. 5). In contrast splanchnic artery occlusion shock produced a marked leukopenia. Our data show that the leukocyte count was markedly decreased at the end (80 min) of reperfusion (Fig. 5). The administration of specific anti E-selectin antibodies significantly ameliorated leukopenia (Fig. 5).

3.6. Histological examinations

Table 1 summarizes the gross lesion, microscopic alteration and leukocyte infiltration in the ileum and

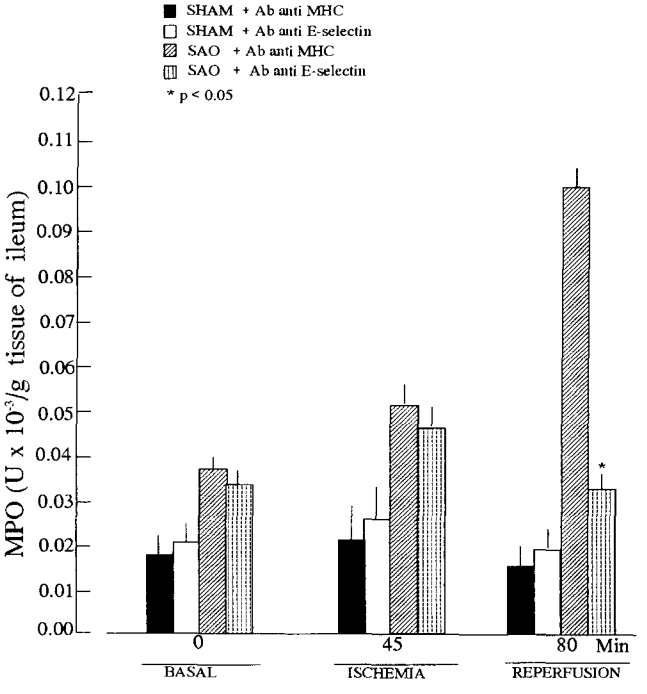

Fig. 3. Effects of anti E-selectin antibodies (2 mg/kg, i.v.) or anti MHC antibodies (2 mg/kg, i.v.) on ileal myeloperoxidase activity of rats subjected to splanchnic artery occlusion shock. Each point represents the mean ± S.D. of seven experiments. * $P < 0.05$ vs SAO + Ab anti MHC.

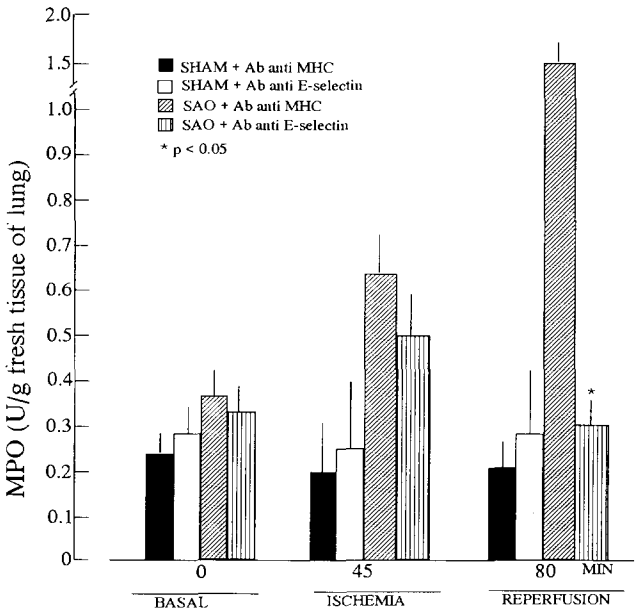

Fig. 4. Effects of anti E-selectin antibodies (2 mg/kg, i.v.) or anti MHC antibodies (2 mg/kg, i.v.) on pulmonary myeloperoxidase activity of rats subjected to splanchnic artery occlusion shock. Each point represents the mean ± S.D. of eight experiments. * $P < 0.05$ vs SAO + Ab anti MHC.

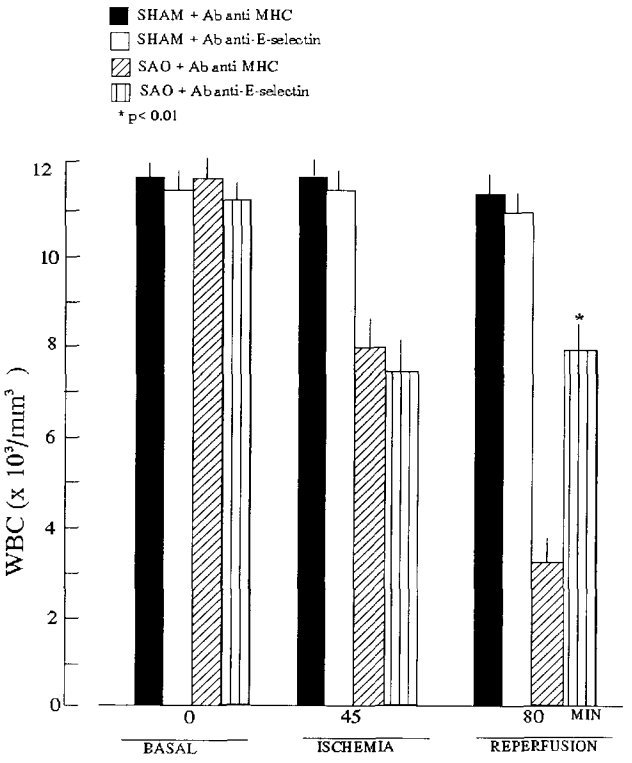

Fig. 5. White blood cell count in splanchnic artery occlusion-shocked rats given either specific anti E-selectin antibodies (2 mg/kg, i.v.) or a non-immune serum (0.3 ml/i.v. dissolved in a phosphate-buffered saline solution at pH 7.4). Bar heights represent the mean ± S.D. from seven experiments. * $P < 0.01$ vs SAO + non-immune serum.

lung of splanchnic artery occlusion-shocked rats treated with either anti MHC antibodies or anti E-selectin antibodies. Splanchnic artery occlusion shock produced characteristic damage of both the ileum (Table 1) and lung accompanied by a marked leukocyte infiltration. In contrast, splanchnic artery occlusion-shocked rats treated with the specific anti E-selectin antibodies showed reduced damage and leukocyte infiltration in the ileum and the lung (Table 1).

Table 1
Effects of Ab anti E-selectin administration on the development of gross lesions, microscopic necrosis and leukocyte infiltration in the ileum of splanchnic artery occlusion-shocked rats

	Gross lesions			Microscopic lesions			Leukocyte infiltration		
	occl.		reperf.	occl.		reperf.	occl.		reperf.
	0	45	80	0	45	80	0	45	80
Sham + Ab anti MHC	0%	0%	0%	–	–	–	–	–	–
Sham + Ab anti E-selectin	0%	0%	0%	–	–	–	–	–	–
SAO + Ab anti MHC	0%	20%	100%	–	+	+ + +	–	+	+ + +
SAO + Ab anti E-selectin	0%	20%	30%	–	+	+ +	–	+	+ +

Gross lesions: presence of haemorrhagic necrosis along the total length of the ileum. Microscopic lesions: heavy (+ + +) marked necrosis is evident everywhere; mild (+ +), necrosis is confined to the tips of the mucosa villi; moderate (+) partial loss of villi, but necrosis is confined to the mucosal layer. Not present = (–). Leukocyte infiltration: heavy (+ + +) very many leukocytes; mild (+ +) many leukocytes; moderate (+) few leukocytes. The antibodies (2 mg/kg, i.v.) or a non-immune serum were injected 3 h before splanchnic artery occlusion shock. The number of rats for each group was ten. SAO = splanchnic artery occlusion.

4. Discussion

E-selectin (previously indicated as endothelial leukocyte adhesion molecule-1) is a structure that causes neutrophils, monocytes and lymphocytes, which are normally free in the circulation, to adhere to the vessel endothelium (Osborn, 1990). A feature of this adhesion is the 'rolling' of the leukocytes, a signalling event that, by altering the conformation of the extracellular binding domain of these molecules, produces the activation of other adhesion molecules belonging to the integrin family (Butcher, 1990; Mackay and Imhof, 1993). This second interaction results in the strong adhesion of leukocytes to the endothelium and provokes the transendothelial migration of leukocytes.

E-selectin has a molecular mass of 115 kDa (McEver, 1991). The binding molecules for E-selectin in neutrophils and monocytes are the L-selectin ligands (Brandley et al., 1990). These ligands are sialylated and fucosilated bearing molecules that are normally present on the membrane surface of monocytes, neutrophils, a subset of memory T-cells and some tumor cells (Brandley et al., 1990). By contrast, E-selectin is not a constitutive ligand of the endothelium, but it may be induced in vitro by the addition of recombinant tumor necrosis factor-α to vascular endothelial monolayers (Pober and Cotran, 1990; Mantovani and Dejana, 1989). It has been suggested that E-selectin may be also present in a soluble form. This idea comes from the observation that activated endothelial cells shed a small proportion of E-selectin in the culture media. In agreement with this hypothesis increased serum levels of soluble E-selectin have been reported in a number of experimental models (Mackay and Imhof, 1993).

Previous findings from our laboratory have suggested that leukocytes are deeply involved in the pathogenesis of splanchnic artery occlusion shock (Canale et al., 1993). However, this preliminary report did not address important questions concerning the involvement of specific adhesion mechanisms in the pathogenesis of splanchnic artery occlusion shock.

In the present study we showed that splanchnic artery occlusion-shocked rats had a decreased survival time accompanied by leukopenia. Furthermore using myeloperoxidase activity as an index of leukocyte infiltration (Mullane et al., 1985) we found that leukocytes are markedly accumulated in the ileum and in the lung at the end of the reperfusion period. There was a good correlation between peak leukopenia and the maximum increase in myeloperoxidase activity: in fact both occurred at 80 min following the onset of reperfusion. We therefore investigated the mechanisms underlying such a phenomenon in splanchnic artery occlusion shock.

Our results clearly indicated that splanchnic artery occlusion shocked rats had increased serum levels of tumor necrosis factor-α. The kinetics of tumor necrosis factor-α appearance in the blood suggests that the cytokine is released mainly during the reperfusion period. Again we found a good relationship between serum tumor necrosis factor-α, the increase in myeloperoxidase activity and peak leukopenia. Moreover, histological studies showed that at the end of reperfusion characteristic damage, mainly haemorrhagic necrosis of both the ileum and lung, occur. In addition, these studies confirmed the accumulation of leukocytes in both organs at the end of reperfusion.

It has been suggested that injection of tumor necrosis factor-α in experimental animals induces leukopenia, leukocyte sequestration and leukocyte adhesion to the endothelium (Munro et al., 1989). Tumor necrosis factor-α is thought to exert control in this process through the ability to increase the expression of endothelial cell-based adherence molecules (Gamble et al., 1985) and more specifically the expression, at least in vitro, of E-selectin (Mantovani and Dejana, 1989). Therefore the key questions that we raised were: does E-selectin play an important role in the pathogenesis of splanchnic artery occlusion shock? Does E-selectin influence leukocyte accumulation in an in vivo model of circulatory shock?

As far as the first question is concerned our present data indicate that passive immunization with specific antibodies raised against E-selectin increases the resistance of rats to the experimental procedures of splanchnic artery occlusion shock. Survival time in the group of rats given an isotype matched monoclonal antibody raised against an MHC antigen was 85 ± 8 min, whereas rats administered the specific anti E-selectin antibodies showed enhanced survival (225 ± 10 min).

Furthermore, the passive immunization with specific anti E-selectin antibodies reduced leukopenia and leukocyte accumulation and protected against the morphological and histological alterations observed in the ileum and in the lung. In light of these data, it could be argued that E-selectin is involved in the pathogenesis of splanchnic artery occlusion shock. In concurrence with this hypothesis recent evidence has suggested that soluble E-selectin levels are significantly increased in splanchnic artery occlusion shock (Altavilla et al., 1994b). Moreover, this molecule significantly contributes, in vivo, to the mechanisms underlying leukocyte accumulation. In splanchnic artery occlusion shock E-selectin seems to be induced by tumor necrosis factor-α, and this latter evidence is in agreement with in vitro data (Mantovani and Dejana, 1989). Finally our data clearly indicate that cell adhesion molecules (i.e. E-selectin) have an important role in leukocyte accumulation in low-flow states and, indeed, the search for

agents that block adhesion events may represent a new avenue in the treatment and management of circulatory shock.

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