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The effects of recombinant human granulocyte-colony stimulating factor on vascular dysfunction and splanchnic ischaemia-reperfusion injury

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1 The aim of our study was to investigate the effects of recombinant human granulocyte-colony stimulating factor in a rat model of splanchnic ischaemia-reperfusion injury.

2 Male anaesthetized rats were subjected to clamping of the splanchnic arteries for 45 min. This surgical procedure resulted in an irreversible state of shock (splanchnic artery occlusion shock; SAO shock). Sham operated animals were used as controls. Survival rate, serum tumour necrosis factor-α (TNF-α), neutrophil count, bone marrow myeloid precursor cells, myeloperoxidase activity (MPO; studied as a quantitative means to assess leukocyte accumulation), mean arterial blood pressure and the responsiveness of aortic rings to phenylephrine (PE, 1 nM – 10 μM) were studied.

3 SAO shocked rats had a decreased survival rate (0% at 4 h of reperfusion, while sham shocked rats survived more than 4 h), increased serum levels of TNF-α (201 ± 10 u ml^{-1}; sham shocked rats = undetectable), neutropenia, enhanced MPO activity in the ileum (0.11 ± 0.06 u $\times 10^{-3}$ g^{-1} tissue; sham shocked rats = 0.02 ± 0.001 u $\times 10^{-3}$ g^{-1} tissue) and in the lung (1.5 ± 0.2 u $\times 10^{-3}$ g^{-1} tissue; sham shocked rats = 0.19 ± 0.05 u $\times 10^{-3}$ g^{-1} tissue) and unchanged bone marrow myeloid precursor cells. Furthermore aortic rings from shocked rats showed a marked hyporeactivity to PE.

4 Administration of recombinant human granulocyte colony stimulating factor (rh G-CSF; 5, 10 and 20 μg kg^{-1} 5 min following the release of occlusion) increased in a dose-dependent manner survival rate (90% at 4 h of reperfusion with the dose of 20 u $\times 10^{-3}$ g kg^{-1}), reduced serum TNF-α (13 ± 5 u ml^{-1}) and MPO activity in the ileum (0.065 ± 0.002 u $\times 10^{-3}$ g^{-1} tissue) and in the lung (0.7 ± 0.03 μg kg^{-1} tissue), improved neutropenia and mean arterial blood pressure but did not modify bone marrow myeloid progenitor cells. Furthermore rh G-CSF, either *in vivo* or *in vitro* (200 nM for 1 h in the organ bath), restored to control values the hyporeactivity to PE. Finally rh G-CSF potently inhibited the activity of inducible nitric oxide synthase in peritoneal macrophages activated with endotoxin.

5 Our results suggest that rh G-CSF protects against splanchnic ischaemia reperfusion injury by a mechanism(s) that does not depend upon its haematopoietic effects.

Keywords: Splanchnic ischaemia-reperfusion injury; recombinant human granulocyte colony stimulating factor (rh G-CSF); impaired vascular reactivity; L-arginine/NO

Introduction

Granulocyte colony-stimulating factor (G-CSF) is a polypeptide growth factor that acts in concert with other endogenous mediators to contribute to the regulation of haematopoiesis and to the control of circulating numbers of leukocytes (Steward, 1993). It acts primarily on cells of neutrophilic lineage, in which it induces proliferation and differentiation of progenitor cells and mobilizes neutrophils from the bone marrow reserve (Demetri & Griffin, 1991). However, it has also been suggested that G-CSF may have important effects outside the haematopoietic system: it induces endothelial cells to migrate and proliferate and this would indicate that it may influence vascular function (Bussolino *et al*., 1989a)

Recombinant human (rh) G-CSF used clinically is produced by the bacterium *Escherichia coli* carrying a cDNA encoding the human gene, and by mammalian cells and it possesses the same activity as the natural factor. This haematopoietic factor does not show species boundary and therefore rh G-CSF has been used in experimental animals (Hollingshead & Goa, 1991).

Occlusion of the major splanchnic arteries followed by reperfusion in anaesthetized rats results in an irreversible circulatory failure and shock (splanchnic artery occlusion shock; SAO shock). This model of shock is characterized by marked hypotension, leukopenia (Squadrito *et al*., 1991), impairment in reticuloendothelial system activity (Sturniolo *et al*., 1989), increased macrophage and plasma levels of thromboxane B_2 (Squadrito *et al*., 1992b) and enhanced blood levels of platelet activating factor (Zingarelli *et al*., 1992) and tumour necrosis factor-α (Squadrito *et al*., 1992a). Furthermore SAO shock is a low-flow state in which leukocyte adhesion to endothelium contributes to the full development of the syndrome (Canale *et al*., 1993).

It has been demonstrated that rh G-CSF has salutary effects on the impaired haemodynamic parameters of experimental animals subjected to bacterial challenge (Haberstroh *et al*., 1991). SAO shock is also characterized by a marked hypotension and vascular dysfunction (Squadrito *et al*., 1994a).

Therefore, the aim of our study was to investigate whether rh G-CSF exerts protective effects on the pathological sequelae associated with SAO shock, a non septic model of shock, by modulating vascular dysfunction. The haematopoietic factor was found to protect against this model of low-flow state and to revert both *in vivo* and *in vitro* vascular dysfunction, thus unmasking a new unknown 'vascular effect' of this haematopoietic cytokine.

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Methods

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^{-1}, i.p.). After anaesthesia, catheters were placed in the carotid artery and jugular vein. Afer midline laparotomy, the coeliac 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 (1,000 μ kg^{-1}, i.v.) and were observed for a 30 min stabilization period before either splanchnic ischaemia or sham ischaemia. SAO shock was induced by clamping both the superior mesenteric artery and the coeliac trunk so as to produce total occlusion of these arteries for 45 min. The clamps were then removed. Following reperfusion the rats were observed for 4 h. Sham shocked rats were subjected to the same surgical procedures as SAO shocked rats except that the arteries were not occluded.

Survival evaluation and arterial blood pressure monitoring

The first group of animals was used to study survival ($n=80$) and arterial blood pressure ($n=28$). Five minutes after the initiation of reperfusion, treated rats received, intravenously, rh G–CSF as bolus injection (5, 10 and and 20 μg kg^{-1}) and control rats received the carrier vehicle. Survival was evaluated for 4 h and expressed either as survival time or survival rate. A group of animals ($n=28$) was also implanted with cannulae (PE 50) inserted into the left common carotid artery and the right jugular vein as described previously (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 presented as mean arterial pressure (MAP) in mmHg. Rats were subjected to the same experimental protocol described above.

Biological assay for TNF-α bone marrow cells and neutrophil count

A third group of animals was used to measure tumour necrosis factor-α (TNF-α), bone marrow cells and neutrophil count. Blood samples were drawn at 0 and 45 min before initiation of reperfusion (by release of the arterial clamp) and at 80 min after reperfusion. The total volume was 1.5 ml and that was replaced with the same volume of 0.9% NaCl. Killing of L929 mouse tumour cells was used to measure TNF-α levels in serum on the basis of a standard assay (Ruff & Gifford, 1980). L929 cells in RPMI 1640 medium containing 5% foetal 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 (0.3 ml, drawn at different time intervals), were made in a medium containing actinomycin D, 1 μg ml^{-1}, 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 Microlisa Autoreader. The percentage of cytotoxicity was calculated as [1-(A490 of sample/A490 of control)] $\times 100$. One unit (u) of TNF-α was defined as the amount giving 50% cell cytotoxicity. The TNF-α content in the sample was calculated by comparison with a calibration curve performed with recombinant murine TNF-α (Nuclear Laser Medicine, Italy). To test whether 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 anti-recombinant TNF-α polyclonal antibodies, but not by control rabbit serum.

Peripheral blood for smears and for quantitation of the absolute number of circulating leukocytes was obtained by tail bleeding at 0 and 45 min before the initiation of reperfusion, and at 80 min after reperfusion. The number of neutrophils (neutrophils $\times 10^3/mm^3$) is presented as mean $\pm$ s.e. The total circulating white blood cell mm^3 was measured with a cell counter (Beckman Instruments Inc. U.S.A.). The percentage and absolute number of circulating white blood cell subsets was determined after a differential count had been done of at least 100 cells/smear.

Bone marrow was obtained from the humerus as previously described (Ulich *et al*., 1989) and at 80 min after reperfusion. The absolute number of nucleated cells/humerus was quantitated with the cell counter. Bone marrow differential was performed on Wrights's-Giemsa stained smears, according to the standard morphological criteria for the rat, as previously described (Hulse, 1964).

Myeloperoxidase activity and isolated aortic rings

A fourth group of animals was used to investigate myeloperoxidase activity and vascular reactivity. Leukocyte accumulation was investigated by measuring the activity of myeloperoxidase (MPO). MPO activity was determined in intestinal mucosa and in the left lung, as previously described (Mullane *et al*., 1985). The samples of intestinal mucosa and left lung were obtained at 0 and 45 min before the initiation of reperfusion (by release of the arterial clamp) and 80 min after reperfusion. The samples were first homogenized in a solution containing 20 mM potassium phosphate buffer (pH 7.4), 0.01 M EDTA, 50 u ml^{-1} protease inhibitor (aprotinin) in proportions of 1:10 (w:v) and then centrifuged for 30 min at $20,000\times g$ at 4°C. The supernatant of each sample was then discarded and the pellet was immediately frozen on dry ice. The samples remained frozen for one entire night before sonication was performed. After being thawed, the resulting pellet was added to a buffer solution consisting of 0.5% hexacyltrimethylammonium bromide (Sigma Chemical Co., St. Louis, MO, U.S.A.) dissolved in 50 nM potassium phosphate buffer (pH 6) containing 30 u ml^{-1} protease inhibitor. Each sample was then sonicated for 1 min at intensity 2 and at a temperature of 4°C by a MSE Soniprep 150 Ultrasonic Disintegrator (MSE Scientific Instruments, Crawley, Sussex, U.K.). After sonication the samples were allowed to chill on ice for approximately 30 min, and then they were centrifuged for 30 min at $40,000\times g$ at 4°C. An aliquot of the supernatant was then allowed to react with 0.167 mg ml^{-1} o-dianisidine dihydrochloride (Sigma Chemical Co.) and 0.0010 % H_2O_2, and the rate of change in absorbance was measured at 405 nm in a microtitre plate reader. MPO activity was defined as the quantity of enzyme degrading 1 μmol of peroxide min^{-1} at 25°C and was expressed in u $\times 10^{-3}$ g^{-1} tissue.

Thoracic aortae were removed 80 min after the onset of reperfusion. The aortae were placed in cold Krebs solution of the following composition (mM): NaCl 118.4, KCl 4.7, $MgSO_4$ 1.2, $CaCl_2$ 1.3, KH_2PO_4 1.2, $NaHCO_3$ 25.0 and glucose 11.7.

Then the aortae were cleaned of adherent connective and fat tissue and cut into rings of approximately 2 mm in length. In some rings the vascular endothelium was removed mechanically by gently rubbing the luminal surface with a thin wooden stick.

Rings were then placed under 1 g of tension in an organ bath containing 10 ml 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 eicosanoids and their metabolites. Developed tension was measured with an isometric force transducer and recorder on a polygraph (Ugo Basile, Comerio (VA), 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 basal tension was readjusted to 1 g, the tissue was exposed to phenylephrine (PE, 100 nM). When the contraction was stable, the function integrity of endothelium was assessed by a relaxant response to acetylcholine (ACh, 100 nM). The tissues were then washed occasionally for 30 min. Concentration-response curves were obtained by cumulative concentrations of phenylephrine (PE; 1 nM to 10 μM) in intact or endothelium-denuded aortic rings. In some experiments rh G-CSF (200 nM) was added to the organ bath 1 h before the rings were contracted with cumulative concentrations of PE. The results (mean $\pm$ s.e.) are expressed as g of tension mg^{-1} tissue.

Nitrite measurement

Peritoneal rat macrophages were cultured in Dulbecco's modified Eagle's medium (DMEM) with L-glutamine (4×10^{-3} M) and 10% foetal calf serum (FCS) as previously described (Szabo *et al*., 1994). Each sample contained 1×10^6 cells ml^{-1}. To induce nitric oxide synthase (iNOS), fresh culture medium containing *E. coli* lipopolysaccharide (LPS, 1 $\mu g\ ml^{-1}$) was added. The dose of 1 $\mu g\ ml^{-1}$ of LPS has been previously shown to induce a marked increase in NO production (Szabo *et al*., 1994). Nitrite accumulation in the cell culture medium was measured after 24 h. To study the effects of rh G-CSF on the production of nitrite, it was added to the medium 6 h after induction of iNOS with LPS. At this time there is no detectable increase in the concentration of nitrite, and agents such as glucocorticoids, that inhibit the induction but not the activity of iNOS, have no effect on subsequent nitrite production (Szabo *et al*., 1994). Nitrite production, an indicator of NO synthesis, was measured in the supernatant of macrophages as previously described (Szabo *et al*., 1994). Nitrite was measured by adding 100 μl of Griess reagent (1% sulphanilamide and 0.1% naphthylethylenediamine in 5% phosphoric acid) to 100 μl samples of cell culture medium. The optical density at 550 nm (OD_{550}) was measured with an microplate reader. Nitrite concentrations were calculated by comparison with OD_{550} of standard solutions of sodium nitrite (5–100 μM) prepared in culture medium.

Drugs

Phenylephrine hydrochloride and indomethacin were obtained from Sigma. rh G-CSF was obtained from Roche Milano S.p.A. Italy. Recombinant mouse granulocyte macrophage colony stimulating factor (rm GM-CSF) and recombinant mouse interleukin-3 (rm IL-3) were purchased from Genzyme Corporations (U.S.A.).

Statistical analysis

The difference between the means of two groups was evaluated with ANOVA followed by Bonferroni's test. *P* values of less than 0.05 were considered to be significant. For survival data, statistical analysis was done with Fisher's exact probability test.

Results

Survival

Sham shocked rats, treated either with the carrier vehicle or with rh G-CSF, survived the entire 4 h observation period (Table 1). In contrast, in rats treated with vehicle, SAO shock produced a profound shock state characterized by a high lethality and no rats survived at 2 h (survival time = 79 $\pm$ 10 min; survival rate = 0%). The administration of rh G-CSF significantly protected in a dose-dependent manner the rats from death induced by SAO shock. In contrast, neither recombinant mouse granulocyte macrophage CSF (rm GM-CSF) nor recombinant mouse interleukin 3 (rm IL 3) modified survival in SAO shock. The most effective dose of rh G-CSF was 20 $\mu g\ kg^{-1}$: in animals that received this dose survival time was 235 $\pm$ 10 min and survival rate was 90% (Table 1). Therefore we used this dose in all the other studies described below.

Table 1 Effect of rh G-CSF on survival in splanchnic artery occlusion (SAO) shocked rats

Treatment	*Survival time (min)*	*Survival animals*	*Survival rate (%)*
Sham + vehicle	> 240	10/10	100
Sham + rh G-CSF (20 $\mu g\ kg^{-1}$)	> 240	10/10	100
SAO + vehicle	79 $\pm$ 10*	0/10*	0
SAO + rm GM-CSF (20 $\mu g\ kg^{-1}$)	83 $\pm$ 11*	0/10*	0
SAO + rm IL-3 (20 $\mu g\ kg^{-1}$)	89 $\pm$ 8*	0/10*	0
SAO + rh G-CSF (5 $\mu g\ kg^{-1}$)	123 $\pm$ 15	0/10	0
SAO + rh G-CSF (10 $\mu g\ kg^{-1}$)	200 $\pm$ 19#	5/10#	50
SAO + rh G-CSF (20 $\mu g\ kg^{-1}$)	235 $\pm$ 10†	9/10†	90

Each point represents the mean $\pm$ s.d. from 10 rats. *$P<0.001$ vs Sham. #$P<0.05$ vs SAO + vehicle. †$P<0.001$ vs SAO + vehicle.

Arterial blood pressure

Occlusion of the splanchnic arteries produced a marked increase in mean arterial blood pressure. Upon release of the occlusion blood pressure decreased substantially and progressively until death (Figure 1). Administration of rh G-CSF significantly blunted the SAO induced reduction in mean arterial blood pressure (Figure 1).

Serum TNF-α

Serum levels of TNF-α were undetectable in sham shocked rats treated either with rh G-CSF or vehicle. TNF-α was also undetectable during the occlusion period. In contrast, serum TNF-α was significantly enhanced at the end of the reperfusion period in SAO shocked rats (201 $\pm$ 10 u ml^{-1}). rh G-CSF substantially decreased TNF-α levels during reperfusion (13 $\pm$ 5 u ml^{-1}; $P<0.001$).

Myeloperoxidase activity

The kinetics of ileal and pulmonary leukocyte infiltration in SAO shocked rats was determined by measuring the myeloperoxidase (MPO) activity in the ileum and lung at 0 and 45 min before the initiation of reperfusion, and at 80 min of reperfusion. MPO levels were significantly increased in the ileum (0.11 $\pm$ 0.06 u $\times10^{-3}\ g^{-1}$ tissue; sham shocked rats = 0.02 $\pm$ 0.001 u $\times10^{-3}\ g^{-1}$ tissue) and in the lung (1.5 $\pm$ 0.2 u $\times10^{-3}\ g^{-1}$ tissue; sham shocked rats = 0.19 $\pm$ u $\times10^{-3}\ g^{-1}$ tissue) at 80 min after reperfusion in shocked rats pretreated with the vehicle.

The administration of rh G-CSF significantly reduced the increase in MPO in both tissues in the ileum to 0.65 $\pm$ 0.002 u $\times10^{-3}\ g^{-1}$ tissue and in the lung to 0.7 $\pm$ 0.03 u $\times10^{-3}\ g^{-1}$ tissue.

Neutrophil count and bone marrow myeloid progenitor cells

The neutrophil count did not change in sham operated rats during the course of the sham experiment when either the vehicle or G-CSF was administered. In contrast, SAO shock produced a marked neutropenia: the neutrophil count was markedly decreased at 80 min of reperfusion (SAO shocked rats + vehicle = 0.5 $\pm$ 0.03 cells $\times10^3/mm^3$; sham shocked rats + vehicle = 2.8 $\pm$ 0.9 cells $\times10^3/mm^3$). Administration of rh G-CSF significantly ameliorated this neutropenia in SAO shocked rats (3.1 $\pm$ 1.1 cells $\times10^3/mm^3$).

Table 2 shows the bone marrow of sham and SAO shocked rats treated either with rh G-CSF or vehicle. Bone marrow

myeloid progenitor cells were not changed by the surgical procedure of SAO shock and rh G-CSF administration did not modify bone marrow myeloid forms.

Contractile response to phenylephrine

Figure 2 shows the contractile force induced by PE (1 nM – 10 μM) in intact aortic rings from sham and SAO shocked rats. SAO shock markedly decreased the contractile response to PE of intact aortae. The administration of rh G-CSF, either *in vivo* or *in vitro*, did not modify the aortic reactivity of sham rats (results not shown). In contrast, administration of rh G-CSF (20 μg kg^{-1}) significantly improved responsiveness to PE in aortae from splanchnic artery occluded rats (Figure 2).

Removal of endothelium did not modify the vascular hyporeactivity of aortic rings from SAO shocked rats (Figure 3): PE produced overlapping effects in aortic rings with (Figure 2) or without endothelium (Figure 3) and endothelium denuded aortic rings collected from rh G-CSF treated rats showed a marked increase in the contractile response to PE (Figure 3).

In endothelium denuded aortic rings from untreated shocked rats G-CSF (200 nM) added for 1 h in the organ bath restored PE-sensitivity to control values (Figure 4), while it did not modify the contractile response to PE of aortic rings collected from sham rats.

Nitrite measurements

To investigate the potential effects of rh G-CSF on inducible nitric oxide synthase activity, the ability of the cytokine to inhibit nitrite production in activated macrophages was tested. Endotoxin significantly increased nitrite production (Table 3). rh G-CSG, when applied 6 h after lipopolysaccharide (LPS) significantly blunted nitrite production by stimulated macrophages. In contrast, neither rm GM-CSF nor rm IL-3 affected LPS-induced NO production (Table 3).

Discussion

G-CSF is a glycoprotein hormone which acts primarily to stimulate proliferation, differentiation and activation of committed progenitor cells of the neutrophil-granulocyte lineage into functionally mature neutrophils (Avalos *et al*., 1990). A recent study has suggested that rh G-CSF possesses beneficial effects on survival and haemodynamics of animals subjected to septic shock (Lundblad *et al*., 1995). However, the beneficial effects of rh G-CSF in this experimental model are ascribed to its multiple biological effects on cells of neutrophilic lineage, but the salutary effects of the haematopoietic cytokine on the vascular parameters also suggest some unknown mechanism(s) of action. Therefore the questions that we raised in the present paper were: (a) is rh G-CSF use restricted to the septic low-flow states, where it is believed that it acts mainly by improving the number and function of neutrophils; or alternatively may it also have beneficial effects in non septic low-flow states ? and (b) are these consequent beneficial effects linked to its multiple biological actions on neutrophils?

To investigate these points, we studied the effects of rh G-CSF on the pathological sequelae associated with splanchnic artery occlusion shock, a non septic model of circulatory shock which is the consequence of a prolonged ischaemia of the splanchnic region (Squadrito *et al*., 1995). Indeed we have shown that SAO shock does not produce release of endotoxin and furthermore polymyxin B, an 'antiendotoxin' antibiotic does not modify the lethal effects and the TNF-α production induced by SAO shock (Squadrito *et al*., 1992a).

Our results clearly indicate that the haematopoietic cytokine significantly increased the resistance of rats subjected to the surgical procedure of SAO shock: in fact the highest dose of rh G-CSF produced 90% survival rate 4 h following the release of occlusion. This is the first study to indicate that the glycoproteic hormone may exert protective effects in non septic ischaemia-reperfusion injury. The effect is specific: indeed neither GM-CSF nor interleukin-3 (both important haematopoietic factors) exerted protective effects in SAO shock. SAO shock is a low-flow state where the leukocyte-endothelium interaction plays a key role. In fact we have previously suggested that leukocytes are deeply involved in the pathogenesis of SAO shock (Altavilla *et al*., 1995a): in fact, specific antibodies raised against E-selection or ICAM-1 ameliorate leukopenia, reduce leukocyte accumulation and increase the resistance of experimental animals to SAO shock (Squadrito *et al*., 1994b; Altavilla *et al*., 1995b).

Our results show that SAO shocked rats have a marked systemic neutropenia and increased leukocyte accumulation in the ileum and in the lung at the end of reperfusion. Furthermore, the administration of rh G-CSF improved neutropenia and reduced leukocyte accumulation in the ileum and in the lung. The improvement in neutrophil count observed in SAO shocked rats treated with rh G-CSF does not seem to be the consequence of the *in vitro* and *in vivo* effects of the cytokine on proliferation and differentation of neutrophilic lineage (Clark & Kamen, 1987; Wang *et al*., 1988); rh G-CSF was administered 75 min before the evaluation of peripheral neutrophil count and bone marrow myeloid precursor cells whereas it has been shown that an intravenous injection of the haematopoietic cytokine causes, after an initial neutropenia of between 5 and 30 min, neutrophilia significantly evident only after 2 h and that peaks between 12 and 24 h (Ulich *et al*., 1988). In agreement with the kinetics of rh G-CSF haematopoietic effect, the recombinant hormone did not cause any increase in neutrophil count in sham rats or any stimulation of bone marrow myeloid precursor cells in either sham or shocked rats. Therefore the rh G-CSF-induced improvement in neutrophil count observed in SAO shocked rats does not depend upon an overflow of neutrophils from the bone marrow or upon stimulation of neutrophil proliferation and differentiation. In contrast, it is possible that in SAO shock rh G-CSF reduces leukocyte accumulation to the peripheral tissue and in turn improves neutropenia. These latter effects could be due to a rh G-CSF-mediated inhibition of TNF-α.

In fact, it has been suggested that TNF-α might also promote the adherence of leukocytes to the endothelium (Gamble *et al*., 1985). Since our treatment succeeded in re-

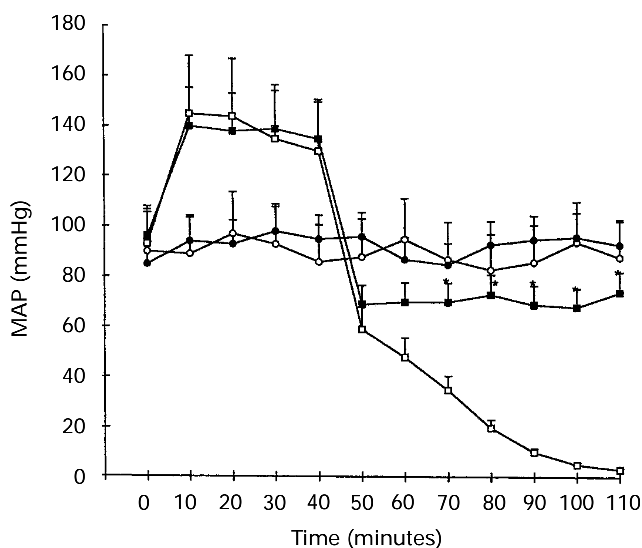

Figure 1 Effects of rh G-CSF (20 μg kg^{-1} h^{-1}, beginning of 5 min after the onset of reperfusion) or vehicle (1 ml kg^{-1} h^{-1} of 0.9% NaCl solution) given intravenously on mean arterial blood pressure (MAP) in rats subjected to splanchnic artery occlusion (SAO) shock. Each point represents the mean and vertical lines show s.e. of seven experiments. (○) Sham + vehicle; (●) Sham + rhG-CSF; (□) SAO + vehicle; (■) SAO + rh G-CSF *$P < 0.001$ vs SAO + vehicle.

Table 2 Bone marrow myeloid progenitor cells in splanchnic artery occlusion (SAO) shocked rats or sham shocked rats

	Sham + vehicle	*Sham + rh G-CSF*	*SAO + vehicle*	*SAO + rh G-CSF*
Myeloblasts	0.86±0.4	0.69±0.3	0.72±0.4	0.62±0.1
Promyelocytes	0.46±0.1	0.77±0.1	0.92±0.3	1.12±0.1
Myelocytes	2.72±0.8	3.23±1.1	3.85±1.8	3.51±0.5
Metamyelocytes	1.12±0.3	1.73±0.8	2.11±1.1	2.21±0.8
Band cells	1.85±0.8	2.14±0.9	3.72±0.4	2.83±1.1
Segmented neutrophils	13.03±2.6	12.92±2.8	11.13±3.1	14.21±4.1
Eosinophils	1.15±0.6	1.44±0.8	1.77±0.9	1.63±0.7
Basophils	0.22±0.1	0.28±0.2	0.34±0.5	0.33±0.9
Monocytes	0.29±0.2	0.79±0.8	0.83±0.7	0.58±0.6
Mast cells	0.42±0.2	0.75±0.2	0.67±0.3	0.99±0.8
Histiocytes	0.59±0.5	0.66±0.3	0.69±0.4	0.72±0.3

Each point represents the mean±s.e. from 10 rats. Experiments were carried out 80 min after reperfusion and 75 min following intravenous administration of either vehicle or rh G-CSF (20 μg kg^{-1}, 5 min after the release of occlusion).

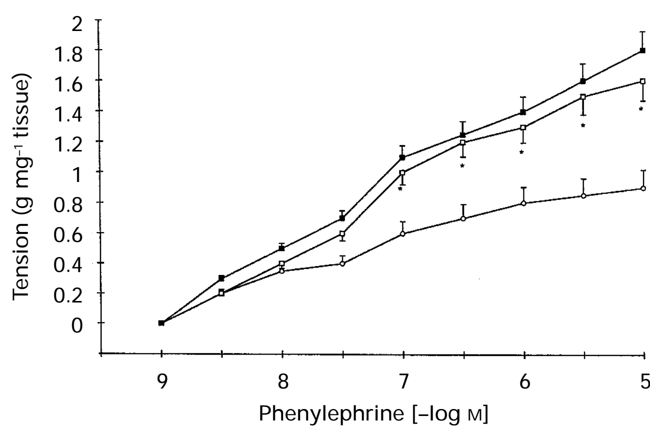

Figure 2 Contractile response to cumulative concentrations of phenylephrine in aortic rings with endothelium from sham occluded or splanchnic artery occluded rats treated with rh G-CSF (20 μg kg^{-1} h^{-1}, i.v. 5 min after the onset of reperfusion) or vehicle (1 ml kg^{-1} h^{-1} i.v. of a 0.9% NaCl solution). (○) SAO + vehicle; (□) Sham + vehicle; (■) SAO + rh G-CSF. Each point represents the mean and vertical lines show s.e. from ten experiments. *$P<0.01$ vs SAO + vehicle.

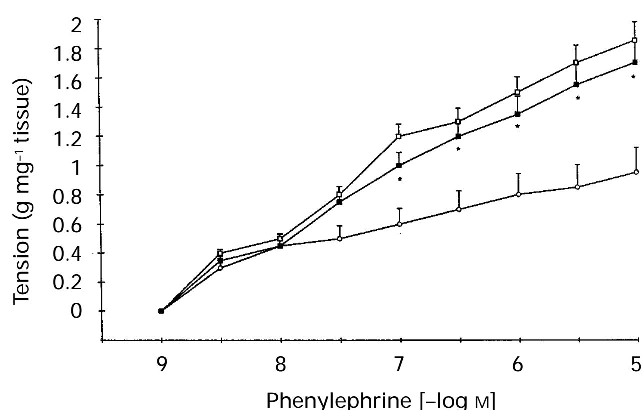

Figure 3 Contractile response to the cumulative concentrations of phenylephrine in aortic rings without endothelium from sham occluded or splanchnic artery occluded rats treated with rh G-CSF (20 μg kg^{-1}, i.v., 5 min after the onset of reperfusion) or vehicle (1 ml kg^{-1} h^{-1}, i.v., of a 0.9% NaCl solution). (○) SAO + vehicles; (□) Sham + vehicle; (■) SAO + rh G-CSF. Each point represents the mean and vertical lines show s.e. of ten experiments. *$P<0.001$ vs SAO + vehicle.

ducing the circulating levels of the inflammatory cytokine, it can also be hypothesized that the beneficial effects of rh G-CSF in intestinal ischaemia-reperfusion injury are, at least in part, due to a decrease in the serum levels of TNF-α (this in turn causing an inhibition of leukocyte accumulation). In agreement with this hypothesis, it has been suggested that G-CSF reduces systemic TNF-α in a model of lipopolysaccharide-induced toxicity (Gorgen *et al.*, 1992). Indeed TNF-α plays a key role in the pathogenesis of SAO shock. In fact, passive immunization with specific antibodies raised against this inflammatory cytokine completely abolished the lethality of this experimental model of shock (Squadrito *et al.*, 1992a).

The mechanisms underlying the irreversible circulatory failure observed in shock are not yet well known. Recently we have shown that in splanchnic-ischaemia reperfusion a marked and composite vascular dysfunction is present in which the L-arginine/nitric oxide (NO) pathway plays an important role (Squadrito *et al.*, 1994a). Indeed aortic rings from shocked rats showed a marked hyporeactivity to phenylephrine and removal of endothelium did not restore the PE-induced contractile response to the values of the sham animal, thus suggesting that smooth muscle cells are involved in the hyporesponsiveness to PE. This complex dysfunction is probably the result of an increase in the endogenous NO produced by smooth muscle inducible NO synthase (Squadrito *et al.*, 1994a). This impairment has been found in both conducting and resistance vessel smooth muscle cells (Squadrito *et al.*, 1994a).

Our data clearly indicates that rh G-CSF significantly improved the vascular dysfunction associated with SAO shock. The effect seems to be a direct phenomenon, since it was observed even when the recombinant cytokine was added in the organ bath containing aortic rings taken from untreated SAO shocked animals. Indeed this result would suggest that rh G-CSF inhibits the inducible NO synthase (iNOS). NO, in fact, is generated following oxidation and cleavage of the terminal nitrogen atom(s) of L-arginine by an enzyme, NO synthase. At least two types of NO synthase have been identified (Moncada *et al.*, 1991). The enzyme found in the endothelial cells is constitutive, Ca^{2+} dependent and releases picomolar amounts of NO for a short period following receptor stimulation. In contrast the enzyme found in the smooth muscle is induced following stimulation with cytokines or endotoxin, is Ca^{2+} independent and releases nanomolar amounts of NO for a long period (Moncada *et al.*, 1991). In order to understand the mechanism by which the hormone interferes with iNOS we performed experiments in LPS-activated macrophages. Our results suggest that rh G-CSF reduces nitrite production, thus strongly indicating that this glycoproteic hormone may inhibit the activity of inducible nitric oxide synthase. This confirms the results obtained when rh G-CSF was added to endothelium denuded aortic rings produced from untreated rats subjected to splanchnic ischaemia reperfusion injury. However, there could be a discrepancy between the two experimental models used to test such an hypothesis (LPS-stimulated macrophages and aortic rings without endothelium) as regards the time

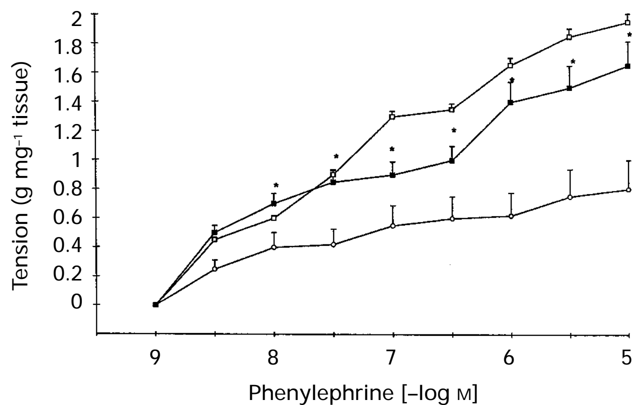

Figure 4 Contractile response to cumulative concentrations of phenylephrine in aortic rings without endothelium from untreated sham occluded or splanchnic artery occluded rats. The rings were incubated for 1 h with rh G-CSF (200 nM) or vehicle (10 μl). (○) SAO + vehicle; (□) Sham + respective vehicle; (■) SAO + rh G-CSF. Each point represents the mean and vertical lines show s.e. of ten experiments. $*P<0.005$ vs SAO + vehicle.

needed to generate NO. In fact NO production occurs in macrophages following 6 h of stimulation with LPS, while the time needed to generate NO in smooth muscle cells, *in vivo*, is lower (45 min of ischaemia following by 80 min of reperfusion). The reason for this discrepancy is that the inflammatory stimuli that activate iNOS is stronger *in vivo* than in *in vitro*. It has been suggested that rh G-CSF activates a Na^+/H^+ exchange mechanism (Bussolino *et al*., 1989b; 1991). Taken together, these findings led us to hypothesize that the triggering of the Na^+/H^+ exchange, may have a role, at least in part, in iNOS inhibition.

Table 3 Effect of recombinant human granulocyte-colony stimulating factor (rh G-CSF) on nitrite accumulation in the supernatant of the cultured macrophages activated with endotoxin

Treatment	*Nitrite* (μM)
Control	1 ± 0.5
LPS (1 μg ml^{-1})	46 ± 2
rh G-CSF (200 nM)	8 ± 1*
rm GM-CSF (200 nM)	51 ± 5
rm IL-3 (200 nM)	66 ± 8

Data are expressed as means ± s.e. of 8 wells from 3–4 independent experiments. Macrophages were stimulated for 24 h with lipopolysaccharide (LPS). rh G-CSF, rm GM-CSF, rm IL-3, were applied 6 h after LPS. $*P<0.01$ vs LPS

In conclusion, we have shown that rh G-CSF protects against the lethal effects in a non septic model of ischaemia-reperfusion injury. In addition, we have demonstrated that these beneficial effects are a consequence of either a reduction of TNF-α or an inhibition of iNOS activity and are not due to its multiple biological actions on neutrophils. Indeed, the haematopoietic hormone possesses a new and unknown positive effect in the failing vascular system that might make it a suitable candidate to be used therapeutically in the treatment of ischaemia-reperfusion injury.

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