Coupling of endothelial injury and repair: an analysis using an in vivo experimental model

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INFLAMMATION IS A KEY RESPONSE to injury; ideally, the inflammatory process should repair tissue structure so that normal function is restored. However, this response lacks specificity, and inflammatory mediators may produce unwanted damage (8, 16). The endothelium is constantly exposed to various serum inflammatory factors (8, 9, 17), most of which have been shown to induce endothelial cell death in vitro (4) and vascular endothelial damage in vivo (4). Some of the inflammatory factors that cause endothelial injury may also stimulate its repair. This can be done either by activating the local molecular cell-repair machinery (12) or by inducing endothelial precursor cell mobilization (3, 7, 13, 18). Circulating angiogenic cells (CACs; also termed early endothelial precursor cells) are cells that appear to stimulate angiogenesis through secretion of growth factors such as vascular endothelial growth factor (15). A link between inflammation-induced endothelial damage and repair is therefore to be expected. Furthermore, the damage to the endothelium itself may promote its own repair; this is suggested by the fact that, in vitro, apoptotic bodies generated from injured endothelial cells induce the maturation of these cells (6).

A coupling between inflammation-induced endothelial damage and repair seems reasonable. However, there is little in vivo evidence to support this phenomenon, and it is important to know how inflammation-induced endothelial damage and repair take place in vivo. An elucidation of the pathophysiology of endothelial injury and repair has been hampered by the lack of an appropriate experimental in vitro model. Most studies have been performed in vivo using isolated endothelial cells in culture without the architecture and matrix of a normal vessel where the complex network of intercellular signals that modulate endothelial cell functions is lacking. Our objective was to provide a better understanding of the coupling of inflammation-induced endothelial damage and repair using an in vivo model. To this end, we have developed a rat model of endothelial damage induced by injecting lipopolysaccharide (LPS) that enables us to analyze the dynamics of endothelial damage and repair.

MATERIALS AND METHODS

Animals. Male Wistar rats (220–250 g) were housed individually and fed a standard diet. The protocol was approved by the Local Animal Welfare Committee. The experimental animals were administered bacterial LPS (Sigma Chemical, St. Louis, MO) at a dose of 1 mg/kg ip at 4, 8, 12, and 24 h before euthanasia (n = 6 for each time point). Control animals received physiological saline. A subgroup of rats that received LPS 8 h before death was pretreated with erythropoietin (EPO; Darbepoetin alpha, AMGEN, Seattle, WA) at a dose of 0.1 μg/kg ip at 12 and 1 h before LPS administration. Control rats received EPO at the same dose and timing. All rats were euthanized using pentothal sodium (Abbott, Redwood City, CA) at a dose of 50 mg/kg, followed by exsanguination by aortic puncture. The thoracic aorta were immediately removed and processed as follows.

Immunohistochemistry. A small fragment of aorta was fixed in 4% paraformaldehyde and subsequently embedded in paraffin blocks. Four-micrometer sections of tissue were used for immunohistochemistry. Endothelial cells were identified by the demonstration of factor VIII-von Willebrand expression using polyclonal antibody (dilution 1/500 of 3.1 g/dl, DakoCytomation, Copenhagen, Denmark), diaminobenzidine (Sigma), and Vectastain Elite ABC (Vector, Burlingame, CA) and counterstained with hematoxylin (Sigma). The microscope was a Leica DM LB2 (Leica Microsystems, Bannockburn, IL), and images of aortic ring sections were obtained with a ×40 objective. We

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have performed histomorphometric analysis of the aorta samples using Image Pro Plus software with ×100 magnification.

**Extraction of aorta cells.** The rest of the aorta (15 mm) was sliced into small pieces (<1 mm³), and cells were dispersed by mechanical maceration under constant saline flow without the use of enzymes; the time taken by this procedure was 15 min. The resulting cell suspension was centrifuged three times at 1,800 rpm for 5 min with physiological saline, and the cell pellet was resuspended in 5 ml of phosphate buffer saline (PBS; Invitrogen, San Diego, CA) with 0.1% bovine serum albumin (BSA; Sigma). The entire procedure of cell extraction from aortic tissue was performed at 4°C.

**Study of endothelial cell apoptosis.** Apoptotic endothelial cells were identified using double fluorescent labeling: specific antibody against rat endothelial cell antigen 1 (RECA-1, 0.5 mg/ml, Monosan, Uden, The Netherlands), which identifies the percentage of endothelial cells of total cells extracted, and annexin V-phycoerythrin (Becton Dickinson), which is specific for cells undergoing apoptosis. Cells obtained from the aorta were incubated with the RECA-1 antibody for 20 min at 4°C and subsequently washed twice in PBS with 0.1% BSA. The cells were then incubated for 20 min at 4°C with IgG1-fluorescein isothiocyanate (FITC) (0.5 mg/ml) as secondary antibody (Becton Dickinson, San Jose, CA), before being washed twice and incubated with annexin V-phycoerythrin (Becton Dickinson) in the presence of annexin buffer containing (in mM) 10 HEPES-NaOH (pH 6.4), 140 NaCl, and 2.5 CaCl₂. After a 10-min incubation period, cells were washed with PBS and resuspended in 500 µl of Cell-Fix (Becton Dickinson). The results were processed and analyzed by flow cytometry using CellQuest software (FACSCalibur, Becton Dickinson).

**Quantification and characterization of circulating angiogenic cells in peripheral blood.** Blood was collected in lithium heparin tubes (LH 34 IU Plus, BD Vacutainer). Peripheral blood cells (100 µl of peripheral blood) were incubated in darkness with 5 µl of the monoclonal antibody against the vascular endothelial growth factor receptor 2 (50 µg/ml, RD Systems, Minneapolis, MN) to identify a subset of functional angiogenic cells in peripheral blood (CACs) capable of reendothelialization (11). After 15 min of incubation, red blood cells were eliminated using a fluorescence-activated cell sorting (FACS) lysis solution (Becton Dickinson) and then was centrifuged at 1,500 rpm during 5 min at 4°C. Cells were then fixed with Cell-Fix (BD Biosciences). The results were analyzed by flow cytometry (FACSCalibur, Becton Dickinson).

In vitro, angiogenic activity of CACs was studied using an Endothelial Tube Formation Assay kit (Cell Biolabs).

**Statistical analysis.** Repeated-measures ANOVA was used to test differences between means over time. Comparisons between two means from different groups were assessed by unpaired t-tests.

**RESULTS**

**LPS induces endothelial cell damage.** Examination of aortic wall samples by immunohistochemistry revealed that 4 h after LPS administration, the endothelium was disrupted, as evidenced by the lack of continuity of the endothelial coating (Fig. 1B). The severity of the lesions was further increased 8 h after LPS administration (Fig. 1C). At 12 h, these abnormalities, although still present, appeared to be less intense than at 8 h. Twenty-four hours after LPS administration, endothelial lesions were no longer apparent. No changes were observed at any time in control samples. When we performed histomorphometric analysis of the aorta samples, we observed that 8 h after LPS administration the number of endothelial cells, using image analysis, decreased to six cells per unit area compared with 11 cells per unit area in the control group.

To quantify the degree of endothelial damage after LPS treatment, cells obtained from the aorta were subsequently analyzed by flow cytometry. Endothelial cells were considered to be those that expressed RECA-1 (Fig. 2A). As assessed by flow cytometry, the proportion of endothelial cells recovered from the aorta of the control animal was 2.35%. In animals treated with LPS, the percentage of endothelial cells significantly decreased at 4 h (from 2.35 ± 0.30% to 1.72 ± 0.28%, P < 0.05; n = 6; Fig. 2B) and continued to drop until 8 h (1.0 ± 0.20%, P < 0.001;
n = 6). Thereafter, the percentage of endothelial cells gradually rose until 48 h, reaching values similar to those observed at baseline (2.35 ± 0.60%; n = 6).

To evaluate apoptosis of endothelial cells, RECA-1 positive cells were evaluated for annexin-V expression, a biological marker of apoptotic death. Figure 3A shows a representative study of apoptosis in endothelial cells. As can be seen in Fig. 3A, of the 2.35% of cells in controls, 7.01% were apoptotic as indicated by the positive annexin labeling. Figure 3B shows the percentage of apoptotic endothelial cells through a period of 48 h following LPS administration. Apoptosis was at a maximum at 8 h after LPS administration, reaching a value of 22.11 ± 1.40%, which is a fourfold increase over baseline values. At 48 h, apoptosis was greater than at baseline; however, post hoc analysis did not display statistical significance.

Interestingly, in aorta exposed to LPS, there was an inverse relationship between the percentage of apoptotic cells and the number of endothelial cells.

Treatment with LPS mobilizes circulating angiogenic cells. CAC mobilization was measured to evaluate the response directed at regenerating damaged endothelium of the LPS-treated rats, which presented an increase in peripheral blood CACs (Fig. 4A). Values were significantly higher at 8 h and remained elevated for 24 h; at 48 h, values were no different from controls. At 4 and 8 h, the increase in the number of CACs in peripheral blood was paralleled by the increase in endothelial cell apoptosis. From 12 to 24 h, apoptosis de-
creased but the number of CACs remained elevated; during this period of time, the total number of endothelial cells returned to normal.

Moreover, after 15 days of culture, more than 90% of CACs were von Willebrand positive and formed tubes (Fig. 4B).

Apoptosis of mature endothelial cells is a main signal for CAC mobilization. To test whether there is a direct relationship between endothelial damage and mobilization of CACs, some of the rats receiving LPS were pretreated with EPO to prevent endothelial cell apoptosis (14). EPO administration produced a significant decrease in the percentage of endothelial cell apoptosis compared with LPS rats that did not receive EPO (Fig. 5A). EPO administration also resulted in a reduction in the percentage of CACs mobilized to peripheral blood (Fig. 5B). In these rats the number of endothelial cells was not different than those in controls (Fig. 5C).

**DISCUSSION**

The aim of the present study was to investigate in vivo a possible coupling between inflammation-induced endothelial damage and repair. We therefore used an experimental rat model of LPS-induced endothelial cell damage. We found that LPS administration induced apoptosis and loss of aortic endothelial cells; these changes were associated with an increase in CACs that persisted until the endothelium was repaired.

The inflammatory response to injury includes the release of mediators that may induce endothelial cell apoptosis. In
most cases the injured endothelial cells can be replaced and the vascular wall will heal without any major clinical consequences. In this study, we have developed an in vivo rat model of LPS-induced endothelial damage that allowed endothelial cell reparation to be evaluated throughout a 24-h period. This model allowed simultaneous analyses of endothelial damage and CAC mobilization to be performed.

Our results show that LPS induces endothelial cell apoptosis with loss of integrity of the endothelial wall. Endothelial damage was quantified by flow cytometry, which was possible because cells from the arterial wall were easily dispersed without the damage that would have been caused by enzymatic digestion.

The percentage of endothelial cells obtained from the aorta of control rats was 2.35 ± 0.30%, a value that is as low as would be expected, given that the endothelium is a thin layer compared with the rest of the arterial wall. The percentage of endothelial cells obtained was constant among the individual animals from the same group, as reflected by the relatively low standard deviation. The percentage of endothelial cells quantified by flow cytometry fell when, as shown by immunohistochemistry, the artery was damaged. Moreover, the percentage of endothelial cells had returned to control levels when microscopically the arterial wall appeared to have returned to normal. Thus, although cell recovery was observed at 24 h, it may well have been completed earlier, between 12 and 24 h. The inverse relationship between endothelial cell apoptosis and the number of endothelial cells in the artery wall suggests that apoptosis is a major mechanism by which the number of endothelial cells decreases in acute LPS exposure.

We observed death by apoptosis in a large number of aortic endothelial cells from animals treated with LPS. Our results confirm reports by others showing that LPS induces endothelial damage (2). It has been suggested that cell apoptosis induced by LPS plays a role in the development of many of the cardiovascular complications associated with sepsis by gram-negative bacteria (5), including acute respiratory distress syndrome (10). It is also important to recognize that in vitro experiments performed by various authors have shown that LPS participates in endothelial wall repair by inducing the synthesis of proteins which serve as mediators of endothelial repair and mobilization of CACs (6, 7, 13, 18). In our in vivo model, LPS produces endothelial damage but also mobilizes CACs. These findings support the role of LPS in the activation of biological mechanisms of endothelial repair (mobilization of CACs) that could not have been observed in an in vitro model. Based on recent studies (6), endothelial injury may produce not only apoptotic microparticles but also stress-induced activation microparticles. Potentially, both types of microparticles may be signals for CAC mobilization. To the best of our knowledge, this is the first in vivo study that demonstrates a coupling between endothelial damage and repair. The concept of a mechanism that is responsible for the coupling of inflammation-induced endothelial damage and repair is not original. A recent report demonstrated in vitro that inflammatory cytokines or reactive proteins responsible for endothelial damage may also stimulate cellular mechanisms that repair the endothelial wall (1). Further in vivo studies are therefore needed to identify potential separate roles of inflammatory mediators and the endothelial damage per se in the mobilization of CACs. In our experiment, the administration of EPO prevented LPS-induced endothelial damage and there was no mobilization of CACs. This makes it difficult to regard inflammatory mediators as the main modulators of cellular repair; if that was the case, the repair process would not be complete until the cessation of the inflammatory process. The results obtained using our in vivo model clearly demonstrated that when the endothelial damage has been repaired, the number of CACs rapidly returns to baseline levels. Besides, serum levels of TNF-α were measured by ELISA at the 8-h time point in rats treated with LPS without EPO and rats treated with LPS plus EPO. The results obtained were 49.8 ± 11.6 and 44.9 ± 5.2 pg/μl for rats without and with EPO treatment, respectively (not significant). In addition to TNF-α, we have found changes in the serum levels of IL-1β that parallel those of TNF-α (data not shown). These results support the hypothesis that endothelial damage rather than inflammatory mediators is the main signal for CAC mobilization and subsequent endothelial repair.

In conclusion, the results of our in vivo experimental model indicate that there is a coupling of inflammation-induced endothelial damage and repair. Apoptosis of endothelial cells appears to be an important event that may synchronize endothelial injury and repair.

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