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Innate immunity has a dual effect on vascular healing: Suppression and aggravation of neointimal formation and remodeling post-endotoxin challenge

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Abstract

Background—Inflammation is important to vascular repair following injury, modulating neointimal proliferation and remodeling. Previously, we have shown that a low-intensity inflammatory response aggravates neointimal formation following balloon and stent injury. The present study examined whether modulation of the extent and timing of nonspecific inflammation mediates the local vascular response in an additive unidirectional or rather a bidirectional fashion.

Methods and results—Rabbits subjected to denudation and balloon injury of the iliac artery were treated with low (1 µg/kg) or high (100 µg/kg) doses of bacterial endotoxin (LPS) immediately after injury, or with early high-dose LPS administered 3 days prior to injury (preconditioning). Neointimal formation at 28 days was significantly increased in the low-dose group ($0.537 \pm 0.059 \text{ mm}^2$) as compared with controls ($0.3 \pm 0.03 \text{ mm}^2$). High-dose LPS did not significantly affect neointimal formation while early high dose significantly reduced neointima (0.296 ± 0.033 and $0.194 \pm 0.025 \text{ mm}^2$, respectively, $n = 12\text{--}14/\text{group}$). Arterial wall and systemically circulating interleukin-1 β levels, and monocyte CD14 activation correlated with neointimal formation. Vascular remodeling was accelerated in animals treated with low- or high-dose LPS while not affected in the preconditioned group. Remodeling index inversely correlated with arterial matrix metalloproteinase-2 levels 6 days after injury.

Conclusions—The extent and timing of nonspecific inflammation that is concurrent with vascular injury can determine different and opposite vascular repair patterns.

Keywords

Inflammation; Vascular injury; Neointima; Restenosis

1. Introduction

Inflammation is a major force in the pathophysiology of atherothrombosis. In addition to its presumed role in the induction, progression and destabilization of the atherosclerotic lesion, the inflammatory response that follows vascular injury is a decisive determinant of the nature of ensuing vascular repair. Inflammatory cells are activated promptly after injury and recruited to the injured vessel wall. The infiltrating inflammatory cells release mediators that modulate smooth muscle cell migration and proliferation as well as further inflammation [1,2]. Elevated inflammatory markers such as total leukocyte count [3,4] and C-reactive protein levels [5,6] are associated with worse outcome after percutaneous coronary artery interventions (PCI). Suppression of innate immunity by various strategies alleviates the inflammatory response following injury and suppresses excessive reactivity, as evidenced by lesser degree of neointimal formation [7–10]. In contrast, events and phenomena that promote systemic inflammation, though not directly related to vascular injury, can exacerbate the reactivity to local vascular injury. We have previously shown, that induction of moderate systemic inflammation with the injection of low-dose LPS increases neointimal formation following superficial and deep vascular injury [9].

Thus, it appears that the convergence of inflammation and vascular injury exacerbates the ensuing proliferation. This may occur by virtue of additive injury or generation of a hypersensitized systemic state. In the former the concomitant effects and potential synergy between inflammatory and injury-released or -produced agents creates more injury, and elicits an expectedly complementary response. In the latter case, the nature of the response is itself different, as the sub-strate on which injury is perceived is changed, heightened and potentially conditioned. In a series of experiments we examined whether modulation of the extent and timing of nonspecific inflammation would mediate the local vascular response in an additive unidirectional pattern or rather a more complicated and at times opposite fashion.

2. Methods

2.1. Vascular injury model

New Zealand White male rabbits (3–3.5 kg, Harlan Laboratories, Jerusalem, Israel) were used in accordance with the guidelines for animal care of the Hebrew University of Jerusalem and National Institutes of Health (USA). Animals were fed standard laboratory chow and tap water ad libitum. Experiments were performed under general anesthesia (xylazine 7 mg/kg and ketamine 40 mg/kg). Heparin (200 U/kg), atropine (0.05 mg), and norfloxacin nicotinate (70 mg) were given and balloon inflation (3-mm angioplasty balloon, Cordis, two 1-min inflations, 8 atm) was performed on the right iliac artery by an investigator blinded to treatment. Animals were randomly assigned to control, saline injections or one of the following treatments with LPS (*Escherichia coli* 0127:B7, Sigma): injection of low-LPS dose (1 µg/kg, low-dose group) immediately after injury, administration of a high-LPS dose (100 µg/kg) 72 h before injury (early high dose, preconditioning) or immediately after injury (high-dose group). Each group consisted of 20 rabbits. Six animals were sacrificed 6 days after injury and 14 at 28 days following injury. LPS doses were chosen following preliminary studies in which incremental doses from 0.1 to 1000 µg/kg were given. The 1 µg/kg dose induced inflammation (increased CD14 expression) without hemodynamic changes while the 100µg/kg dose induced a toxic non-lethal state.

After euthanasia with pentothal, arteries were perfusion-fixed *in situ* with 150mL of 4% formaldehyde solution (pH 7.4) for morphometric analysis. The arterial segments were embedded in paraffin and cut at 8–10 sites (600 µm apart). Sections of 6µm were mounted and stained with verHoeff's elastin stain, hematoxylin and eosin, or modified Movat pentachrome.

Arterial blood was collected into citrate-containing tubes 24 h before the procedure and at 1, 2, 6, 14, 21 and 28 days after injury.

2.2. Morphometric analysis

Histologic sections were examined microscopically by an investigator blinded to treatment. Eight to 10 sections in each slide were analyzed by computerized morphometry (NIH Image). The section with the greatest luminal narrowing by neointima was analyzed as previously described [11]. The residual lumen, the area bounded by the internal elastic lamina ("original" lumen), and the area circumscribed by the external elastic lamina (total arterial area) were measured directly. The degree of neointimal thickening was expressed as the ratio between the area of the neointima and the original lumen (% stenosis) and as the ratio between the neointimal area to the area of the media (N/M). Remodeling of the injured artery was calculated by dividing the cross-sectional area within the external elastic lamina (EEL) by the area within the EEL of the uninjured contra-lateral artery.

2.3. Cytokine and monocytes activation

2.3.1. Arterial IL-1 β production and transcription—Cytokine production was followed in separate groups of animals. Animals were sacrificed 6 days post-angioplasty. Arteries were homogenized in collagenase buffer and extracted IL-1 β was measured using commercial ELISA kits (R&D Systems) the data was normalized to protein content measured by BCA commercial ELISA kit (Sigma). Plasma was separated and stored at -70°C until further analysis. IL-1 β levels were measured in thawed triplicates with a commercial ELISA kit (Biosource International).

2.3.2. Matrix metalloproteinases activity—Arteries were homogenized in collagenase buffer and their supernatant analyzed for collagenase activity. Samples were separated on gelatin-impregnated (1 mg/mL: Difco) SDS 8% polyacrylamide gels under nonreducing conditions, shaken 30 min in 2.5% Triton X-100 (BDH), incubated in collagenase buffer (16 h, 37°C), and stained with 0.5% Coomassie G 250 (BioRad) in methanol/acetic acid/ H_2O (30:10:60). Band intensity was determined by computerized densitometry (Molecular Dynamics type 300 A).

2.3.3. CD14 expression on monocytes—CD14 is a protein essential for LPS-induced cellular activation. CD14 expression is increased in activated monocytes. The activation status of monocytes was examined in triplicates by flow cytometry. Anti-coagulated blood (200 μL) was incubated for 30 min (4°C , in the dark) with mouse anti-human RPE-conjugated anti-CD14 (DAKO). FACS lysing solution (1:20 dilution) was added for 15 min. The residual cells were washed (X1500 RPM, 5 min, 4°C) in fluorescence activated cell sorting (FACS) medium (PBS, 1% BSA, 0.02% sodium azide) and suspended in 1mL FACS medium for flow cytometry. Monocytes were identified according to their forward and side-scattering properties as well as fluorescence. The effect was tested at different time points following IV injection of LPS (1 and 100 $\mu\text{g/kg}$) and at equivalent saline volumes to rabbits. Blood was drawn at baseline and after 1, 2, 6, 14, and 21 days.

2.4. Statistics

Results were expressed as mean $\pm$ S.D. Statistical differences between groups in the *in vitro* experiments were assessed by the ANOVA test, using the InStat software. Values of $P < 0.05$ were considered significant.

3. Results

3.1. Effect of LPS on balloon injury-induced intimal hyperplasia

Iliac balloon injury resulted in abundant concentric neointimal formation (Fig. 1). Neointimal area in the control group was $0.3 \pm 0.03 \text{ mm}^2$, luminal area was $0.13 \pm 0.01 \text{ mm}^2$, luminal stenosis was $69 \pm 8.6\%$, and iliac neointimal area to medial area ratio was 1.18 ± 0.1 . Treatment with a low dose of LPS ($1 \mu\text{g/kg}$, LD) increased neointima formation, stenosis and neointima to media ratio, to $0.537 \pm 0.059 \text{ mm}^2$, $87 \pm 8\%$ and 1.76 ± 0.11 , respectively. Administration of a 100-fold higher dose ($100 \mu\text{g/kg}$) 72 h before injury (early high dose, EHD), and immediately after injury (high-dose group, HD), significantly reduced intimal area and luminal stenosis in the iliac arteries, $56 \pm 5\%$ and $0.19 \pm 0.025 \text{ mm}^2$, and $60 \pm 8\%$ and $0.296 \pm 0.033 \text{ mm}^2$, respectively (Fig. 1). Arterial remodeling index in the control group was 1.28 ± 0.24 ; following LPS administration significant positive remodeling was observed in the LD and HD groups, 2.54 ± 0.214 and 1.76 ± 0.28 , respectively. In the EHD group, no significant change in remodeling was observed with a remodeling index of 1.15 ± 0.24 .

3.2. Arterial IL-1 β

A significant IL-1 β response was observed in animals treated with low-LPS dose ($1 \mu\text{g/kg}$) in comparison to the control group. The response appeared both in the injured arterial wall as well as in the contra-lateral, non-injured artery. A milder response was observed in animals treated with high-LPS doses (EHD and HD groups, Fig. 2a). To examine the predictive value of an early nonspecific stimulation of the innate immune system on late stenosis, the arterial IL-1 β level 6 days after injury was plotted against stenosis extent at 28 days. A strong positive correlation was demonstrated ($r^2 = 0.887$), and the highest increase in IL-1 β was associated with the maximal stenosis (Fig. 2b). A similar correlation was observed when IL-1 β levels were compared to the *N/M* ratio (data not shown).

3.3. Plasma IL-1 β

IL-1 β levels in plasma were increased 2 days after pretreatment with high-LPS dose (EHD) with a further increase observed at the day of injury (Fig. 3a). A significant increase of IL-1 β levels, 2 days after injection, was observed in the LD group in comparison to the control, HD and EHD groups, 522 ± 27 , 370 ± 22 , 371 ± 46 and $332 \pm 35 \text{ pg/mL}$, respectively. IL-1 β levels remained higher in the low-dose LPS group. Injection of LPS to non-injured animals induced a higher IL-1 β response in rabbits plasma, with a stronger response in those treated with a low-LPS dose. Levels at day 6 in animals treated with high and low dose without injury in comparison to the control group were, 1321 ± 100 , 1276 ± 74 and $191 \pm 53 \text{ pg/mL}$, respectively (Fig. 3b).

3.4. Arterial matrix metalloproteinases (MMPs)

Injection of low-dose LPS on the day of injury significantly reduced arterial MMP-2 activity in comparison to the control group (table in Fig. 4). The reduction was less pronounced in the HD group with no suppression in arterial MMP-2 levels observed in the EHD group. MMP-9 levels in the iliac arteries were below the detection limit at all treatments. To examine the predictive value of early nonspecific stimulation of the innate immune system on late remodeling, arterial MMP-2 levels at 6 days after injury were correlated with the remodeling rate (EEL of the injured artery/EEL of contra-lateral artery) at 28 days. A strong inverse correlation was demonstrated for MMP-2 ($r^2 = 0.94$, Fig. 4). The reduction in MMP-2 levels was not limited to injured artery and was observed also in contra-lateral non-injured iliac arteries (Fig. 4).

3.5. Monocyte activation

Activation of circulating monocytes was significantly increased by a low dose of LPS (1 μ g/kg, at the time of injury) in comparison to injured control animals. 24 and 48 h after vascular injury, there was a $150 \pm 20\%$ increase in CD14 expression after LPS low-dose treatment. In contrast, after early and concurrent high-dose treatments of LPS (100 μ g/kg, 72 h prior to injury or at the day of injury) massive reduction was observed at 24 h and at 48 h after injection (Fig. 5a). Moreover, changes in monocyte number were also observed in non-injured rabbits following treatment. Increase of CD14 expression in the LD groups was observed in comparison to a decrease in the HD group 24 h after injection and a massive increase after 6 days. No significant changes were observed in both control and non-injured rabbits (Fig. 5b).

4. Discussion

The contribution of “nonspecific” inflammation to vascular repair, and to the outcome of vascular intervention and restenosis raises several basic questions regarding the dose–response and time–response between inflammation and repair. The present study examined the effect of different regimens of nonspecific modulation of innate immunity on inflammatory activation and vascular reparation following balloon injury. It appears that innate immunity may regulate vascular repair in opposite directions; nonspecific low-intensity inflammation provokes stronger neointimal formation and increases remodeling, while high-intensity stimulation, especially if applied prior to vascular injury, leads to suppression of the inflammatory response that modulates excessive reparation, and is manifested with reduced neointimal formation and expansive remodeling.

Systemic infection with cytomegalovirus (CMV) is associated with increased neointimal formation following balloon injury [12]. Such increased reparative response is suppressed by nonspecific anti-inflammatory therapy [13], suggesting that the inflammatory response that modulates vascular healing is nonspecific. In a previous study in balloon and stent-injured rabbits, low-dose LPS treatment increased neointimal formation [14]. IL-1 β levels correlated with neointimal hyperplasia, further supporting the “nonspecific inflammation” theory [14]. The present study verified our previous findings; low-dose LPS therapy activated blood monocytes, increased local and systemic IL-1 β expression, and increased neointimal formation. Higher doses of LPS had an opposite effect on innate immunity. Monocyte activation and IL-1 β expression were suppressed with subsequent reduction of neointimal formation, especially when high-LPS treatment was applied 72 h before injury. Thus, innate immunity effect on vascular healing can be bi-modal, and the time–response is not less important than the dose–response.

Exposure to high non-lethal doses of bacterial endotoxin results in a state of immune hyporesponsiveness [15,16]. This hyporesponsive innate immune state (referred by some as preconditioning) modulates cardiovascular healing following myocardial ischemia with reduced TNF- α expression and reduced infarct size [17]. Our studies in non-injured rabbits demonstrate the hyporesponsiveness of the innate immunity. Following an initial innate immune stimulation that is marked by an early peak of plasma IL-1 β , there is a marked reduction in monocyte activation as determined by suppression of monocyte CD14 expression. The changes that are seen after high-dose LPS administration are further modulated by vascular injury: IL-1 β peak levels are reduced, and monocyte suppression is prolonged. When injury occurs at the peak of immune hyporesponsiveness, i.e., at 72 h after LPS, vascular healing is preconditioned with a marked suppression of neointimal formation.

The correlation between arterial IL-1 β levels at 6 days after injury with neointimal growth after 28 days (Fig. 2) provides a new physiologic indicator for the inflammatory response evidenced by neointimal formation. IL-1 β is not only a marker but rather a mediator of SMC proliferation

[18], that is important in neointimal formation [19]. However, the correlation was not precise since there was no marked reduction in IL-1 β arterial levels in the preconditioned group to correlate with the significant reduction in neointimal formation observed in this group. This may result from additional mediators such as CD14 activation [14], from the systemic changes in IL-1 β expression (Fig. 3a) that are more pronounced than local changes, or from the single time point sampling as well as the lack of arterial IL-1 β time points.

Vascular healing following injury is comprised from neointimal formation as well as vascular remodeling, both affecting luminal area. MMP-2 holds a major role in constrictive remodeling and its inhibition post-injury is associated with expansive remodeling [20]. Previous studies have shown activation of endothelial MMP-2 in response to LPS [21]. Our results, in the *in vivo* model utilized, are not in accord with those previous findings; low-dose LPS was associated with suppressed MMP-2 activity, with amelioration of this effect in the HD LPS group. MMP-2 activity in the EHD group was comparable with that recorded in non-treated controls. While it is not clear why the inflammatory stimulation induced by LPS suppresses MMP-2 activity, our results further support the correlation between inflammation, low MMP-2 activity and expansive remodeling.

A recent study of the genetic inflammatory factors that predict restenosis after coronary interventions support the role of inflammation in modulating the vascular repair [22]. This corroborates the results of elevated inflammatory markers that are associated with worse outcome after PCI [23–25]. Our findings enable the dissection of the inflammatory response and its effects on vascular repair. There is more than a single pattern of modulation, and inflammation may hold both stimulatory and suppressive bearings on vascular healing. The intensity of inflammation and its timing in relation to vascular injury may modulate inflammatory reactions, which may translate to opposite effects on vascular reparation. Low-intensity inflammation may lead to enhanced neointimal formation and remodeling while excessive inflammation may result in an opposite response.

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Fig. 1. Hypercholesterolemic rabbit iliac artery 28 days after balloon injury. Photomicrographs of verHoeff's tissue elastin staining full-sized sections from untreated (a) and rabbit treated with LPS: low (b) high (c) and early high doses (d). Bar graphs show (e) extent of stenosis (%), and (f) the extent of neointima (and neointima to media ratio) following treatment ($n = 12$ in each group, $*P < 0.05$, $**P < 0.01$). Values are mean $\pm$ S.D.

Fig. 2.

Arterial IL-1 β levels after injury and relation to % stenosis. (a) IL-1 β levels in injured and contra-lateral iliac arteries at 6 days following balloon injury. ($n = 6$ in each group, $*P < 0.05$, low dose vs. control, early high dose and high dose, high dose vs. control.) IL-1 β levels following injury are increased in both the injured and the contra-lateral artery in animals subjected to low-dose LPS. A smaller response is observed in animals treated with high-dose LPS at the time or 3 days prior to injury (early high dose). (b) Correlation between IL-1 β plasma level at 6 days after injury and % stenosis. Values are mean $\pm$ S.D.

Fig. 3. Plasma IL-1 β levels after balloon injury. (a) Injured iliac artery and (b) non-injured group. Note to the high increase in IL-1 β level in low-dose group 48 h after treatment, IL-1 β levels remain higher throughout the 21 days ($n = 6$ in each group, $*P < 0.05$). Values are mean $\pm$ S.D.

(a) MMP-2 (pg/mg)				
	Control	Low dose	High dose	Early high dose
iliac injury	12±1	5.8±0.3	8±0.25	12.3±0.5
iliac contra-lateral	8.4±0.3	6.3±0.3	6.5±0.7	9.45±1

Fig. 4.

Arterial MMP-2 levels after injury and relation to remodeling. (a) MMP-2 levels in injured and contra-lateral iliac arteries at 6 days following balloon injury. ($n = 6$ in each group, $*P < 0.05$, control and early high dose vs. low and high.) MMP-2 levels following injury are decreased in both the injured and the contra-lateral artery in animals subjected to low-dose LPS. A smaller response is observed in animals treated with high-dose LPS. (b) The correlation between matrix metalloproteinase (MMP-2) levels 6 days after balloon injury and arterial remodeling following LPS treatment at 28 days after injury. Decreased MMP-2 response is associated with increased remodeling. Values are mean $\pm$ S.D.

Fig. 5.

Monocyte activation after balloon injury. Line graph of a representative flow cytometry analysis for CD14 expression (as percentage change from baseline levels) on peripheral blood monocytes in control (saline injected) and LPS treated rabbit. (a) Injured group and (b) non-injured rabbit group ($n = 12$ in each group, $*P < 0.05$ control vs. low, high and early high, high vs. low). Values are mean $\pm$ S.D.