

Leukocyte recruitment and expression of chemokines following different forms of vascular injury

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Abstract: Inflammation plays a central role in restenosis following coronary intervention. Recent human and animal data suggest important differences between the inflammatory responses to simple balloon angioplasty compared with stent implantation. To investigate the mechanisms of these differences, New Zealand white rabbits underwent bilateral iliac artery balloon denudation. Half received intravascular stents. Arteries were harvested at three, seven and 14 days for immunohistochemistry, and 4 hours, 8 hours and 14 days for chemokine mRNA analysis. Leukocyte content was quantified utilizing immunohistochemistry (RPN357, monoclonal antibody (mAb) against rabbit neutrophil; RAM-11, mAb against rabbit macrophage). We analyzed the mRNA levels of the chemokines monocyte chemoattractant protein 1 (MCP-1) and interleukin 8 (IL-8) through semi-quantitative polymerase chain reaction. We demonstrated the spatial pattern of MCP-1 mRNA levels through *in situ* mRNA hybridization. In balloon-injured arteries, leukocyte recruitment was confined to early neutrophil infiltration. IL-8 and MCP-1 mRNA levels peaked within hours and were undetectable at 14 days. In contrast, in stented arteries, early neutrophil recruitment was followed by prolonged macrophage accumulation. IL-8 and MCP-1 mRNA levels peaked within hours but were still detectable 14 days post injury. Conclusions: In contrast to balloon injury, stent-induced injury results in sustained chemokine expression and leukocyte recruitment. These data may have important implications for antirestenotic strategies.

Key words: chemokines; inflammation; restenosis; stents

Introduction

Our understanding of the pathophysiologic response to vascular injury is largely derived from the study of animal models involving simple balloon denudation of vascular endothelium. Yet the majority of patients undergoing percutaneous coronary intervention now receive an intracoronary stent. This change in clinical practice derives from the reduction in restenosis seen with stents¹ and the more predictable immediate result seen with stent placement. In the new era of drug-eluting stents, their use will undoubtedly increase even more. It is increasingly clear that inflammation plays a central role in restenosis following coronary intervention. Recent human and animal data indicate that there are important differences between the inflammatory response to simple balloon angioplasty and the deep and chronic injury associated with stent implantation. Little is known about the mechanisms of leukocyte recruitment that determine differences between balloon and stent injury, and that might, therefore, be exploited to alter vascular repair.

Leukocyte recruitment following vascular injury occurs at sites of endothelial denudation in the presence of platelets and fibrin. Central to recruitment of leukocytes to areas of injury, chemokines are a group of chemoattractant cytokines produced by a variety of somatic cells including smooth muscle cells endothelial cells, and leukocytes. One such chemokine of the CC class, monocyte chemoattractant protein 1 (MCP-1), participates in the recruitment of monocytes (as well as basophils and certain activated T cells).² Of particular importance in recruitment of neutrophils, is the C-X-C chemokine interleukin 8 (IL-8).³ The hypothesis of this study was that the kinetics of inflammatory cell recruitment and infiltration differ markedly between stent- and balloon-induced injury, and that these differences should be demonstrable in cellular responses as well as chemokine expression.

We now report two series of experiments. In the first, we studied the temporal and spatial patterns of leukocyte recruitment in balloon-injured or stented rabbit iliac arteries. In the balloon injury model we observed only a transient influx of neutrophils and no macrophages. In contrast, in stented vessels we saw early infiltration of neutrophils followed by sustained accumulation of monocytes. In the second experiment, we determined mRNA levels of the chemokines MCP-1 and IL-8 at sites of vascular injury utilizing both semi-quantitative reverse transcriptase polymerase chain reaction and *in situ* hybridization. In balloon injury, we found transient (hours) presence of mRNA for MCP-1 and IL-8. In contrast, in stented arteries, there was sustained presence of mRNA for IL-8 and MCP-1 as late as 14 days. These data suggest that there are fundamental

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differences in the vascular biologic response to balloon-versus stent-induced injury. These differences may have implications for the development of local or systemic pharmacologic anti-inflammatory strategies against restenosis.

Methods

Animal care

This study was conducted in research facilities accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) and licensed by the United States Department of Agriculture (USDA) to conduct research in laboratory animals in compliance with the Animal Welfare Act, USDA regulations and National Research Council (NRC) guidelines.⁴⁻⁶ Animal activities described in this protocol were subject to review and approval by the Institutional Animal Care and Use Committee (IACUC) of MIT. Animal husbandry, diet, water and environmental conditions were performed in compliance with NRC guidelines.⁵

Surgical and tissue retrieval techniques

New Zealand white rabbits (Covance Products, Denver, PA, USA), weighing 3–4 kg, were housed individually in steel mesh cages and fed rabbit chow and water ad libitum. Under anesthesia with intramuscular ketamine (Aveco Co., Fort Dodge, IA, USA) 35 mg/kg and xylazine (Miles Inc., Shawnee Mission, KS, USA) 15 mg/kg, both femoral arteries were exposed and ligated. Bilateral femoral arteriotomies were performed and a 3 Fr balloon catheter (Baxter HealthCare Corp., Irvine, CA, USA) passed retrograde into the abdominal aorta and withdrawn in the inflated state three times in order to denude the iliac artery endothelium bilaterally. In half the animals, after balloon denudation a 10-mm-long endovascular stent mounted coaxially on a 3-mm angioplasty balloon (Guidant/ACS) was passed retrograde via the arteriotomy into each iliac artery and expanded with a 15-second inflation at a pressure of 8 atm. Rabbits receiving stents were started on aspirin water (Sigma Chemical Co. St Louis, MO, USA, 0.07 mg/ml) one day prior to the procedure. In addition, rabbits receiving stents also were given a single intravenous bolus of standard anticoagulant heparin (100 U/kg; Elkins-Sinn Inc., Cherry Hill, NJ, USA) at the time of deployment to limit acute stent thrombosis. Rabbits undergoing mRNA analysis underwent identical procedures with the exception that injury was performed unilaterally to preserve the contralateral artery as an internal control.

Rabbits undergoing immunohistochemical analysis were euthanized at 3 ($n = 15$ arteries), 7 ($n = 12$ arteries), and 14 days ($n = 8$ arteries) after surgery. Anesthesia was administered as above, the caudal vena cava was opened, and pressure perfusion performed with Ringer's lactate solution (300 ml) through left ventricular puncture, followed by 4% paraformaldehyde (PFA) for 10 min at 100 mmHg. The iliac arteries were excised and placed in a solution of 4% PFA for 24 h. Arteries undergoing mRNA analysis were harvested after 4 h ($n = 3$), 8 h ($n = 3$), and 14 days ($n = 3$) post injury. Stents were dissected from the tissue preserving both neointima on the luminal side of the stent as well as the medial and adventitial layers, and then snap frozen in liquid nitrogen.

Histological and immunocytochemical analysis

Specimens were embedded in methyl methacrylate mixed with *n*-butyl methacrylate (Sigma Chemical Co.).^{7,8} Tissue and cell structures were identified in histological sections by staining with verHoeff's tissue elastin stain, or hematoxylin and eosin. The luminal surface was examined for adherent leukocytes categorized as either monocytoid or polymorphonuclear under 600 \times magnification.

Species-specific antibodies were utilized to immunocytochemically identify rabbit macrophages (RAM 11; DAKO Corporation, Carpinteria, CA, USA) and rabbit neutrophils (RPN 3/57; Serotec Inc., Raleigh, NC, USA). Rabbit spleen was used as a positive control. As RPN 3/57 IgG also identifies rabbit thymocytes, identification of cells as neutrophils was confirmed by examining serial sections for characteristic morphology under hematoxylin and eosin stain (multilobulated nuclei and granuloctytic cytoplasm) and immunocytochemically stained sections. Standard immunocytochemical protocols were used in conjunction with heat-induced epitope retrieval as previously described.⁸ Sections were heated to 80°C in Target Retrieval Solution (DAKO Co.), incubated with the primary antibody followed by a biotinylated species-specific secondary antibody (Vector Laboratories, Burlingame, CA, USA), and stained with avidin-biotin peroxidase or avidin-biotin-alkaline phosphatase followed by 3,3-diaminobenzidine (Sigma Chemical Co.) or alkaline phosphatase (Vector Laboratories Inc.). Overall cell density was calculated by dividing the number of nuclei by the intimal or medial area.

Semi-quantitative reverse transcriptase PCR

Injured iliac and normal carotid arteries were harvested, the stents were harvested, the stents immediately removed, and tissues snap frozen in liquid nitrogen. RNA was prepared from harvested vessels by high-speed homogenization (Kinematica, Lucerne, Switzerland) followed by acid-phenol extraction using the RNazol reagent (TelTest, Inc., Friendswood, TX, USA). cDNA was prepared from 5 μ g RNA using reverse transcriptase (Superscript II; Gibco BRL Grand Island, NY, USA). PCR amplification of MCP-1, IL-8, and 36B4 was performed using ³²P-dCTP tracer in an Idaho thermal cycler in microcapillary tubes for 33 cycles. PCR primers were selected by direct inspection of gene sequences to yield 200–400 base pair PCR products. Primer sequences are as follows:

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MCP-1 5' CCAACCTGGACAAGAAATGCAGA
MCP-1 3' AGAACTGGGGTTCACAGAGGGAA
IL-8 5' ATGACTTCCAAGCTGGCCGTGC
IL-8 3' TATGACTCTTGCTGCTCAGCCCT
36B4 5' TCATTGTGGGAGCAGACA
36B4 3' GGAGAAGGGGGAGATGTT
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Positive controls for MCP and IL-8 were prepared from rabbit splenocytes stimulated *in vitro* with lipopolysaccharide (LPS). Triplicate PCR products were separated on 2% agarose gels in TAE buffer. Gels were inspected under UV light for product purity, and sections of the gel corresponding to the range from 100 to 700 bp were cut. Amplified PCR products were then blotted onto Nytran-N charged nylon membranes (Schleicher and Schuell, Keene, NH, USA). Blots were exposed to PhosphorImager cassettes (Molecular Dynamics, Amersham, Arlington Heights, IL, USA).

Background for each band was determined by local median using PhosphorImager package software. The mean signal intensity for each triplicate was calculated. A normalized ratio of chemokine message to the reference gene 36B4 was calculated. A ratio of normalized chemokine mRNA levels in the injured artery compared with the normalized chemokine mRNA levels of the uninjured (control) artery was calculated.

Standard error for values was calculated using the package software.

In situ hybridization

Animal and tissue preparation

Two male 3–5 kg New Zealand white rabbits were subjected to bilateral femoral stent implantation with methods described above ($n = 4$). The femoral arteries ($n = 2$) of an uninjured rabbit were used as sham injury control. One rabbit was sacrificed at 4 h and the other at 14 days after injury. The femoral arteries were dissected and the stent removed, fixed in 10% neutral buffered formalin for 24 h and paraffin embedded. MCP-1 hybridization was performed on 5 μm sections cut from the arteries. This process resulted in loss of the neointima for purposes of staining.

MCP-1 probe preparation

Full-length MCP-1 cDNA (inserted in the BlueScript plasmid) was the kind gift of Teizo Yoshimura. The plasmid was purified using the Qiagen Plasmid Mega Kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer's instructions. The MCP-1 insert was cut from the plasmid with *Xba* and *Hind*III endonucleases and extracted using low-melting-point gel electrophoresis extraction technique.

In situ hybridization

MCP-1 cDNA (2.55 μg) was labeled with digoxigenin (Roche DIG Chem-Link Labeling kit; Roche Diagnostics, Indianapolis, IN, USA) according to the manufacturer's specification and used as a probe for in situ hybridization. Digoxigenin-labeled positive control oligo (ALU) probe and negative control probe obtained from Kreatech (Amsterdam, The Netherlands) were used as positive and negative controls. Three 5 μm sections were cut from each femoral artery. The tissue sections were deparaffinized and rehydrated with TBS buffer and then treated with proteinase K (DAKO Corp.) for 5 min at 37°C. The sections were then acetylated by immersion into a solution of 0.25% acetic anhydride, 0.1 ml/l triethanolamine and 0.9% sodium chloride for 10 min at room temperature. After washing twice with 2 \times SSC, the sections were prehybridized with Sigma prehybridization solution (100 μm per section) at room temperature for 3 h. The digoxigenin-labeled MCP-1 probe was heated to 95°C for 10 min and then cooled on ice rapidly for 30 s. The MCP-1 probe was diluted with Sigma hybridization solution to a concentration of 10 ng/l. Hybridization was performed by applying 25 μl of the MCP-1 hybridization solution to the tissue sections and incubated at 65°C for 18 h. Equal amount of the digoxigenin-labeled positive and negative control probes (Kreatech) were applied to the sections and hybridized under the same conditions. After hybridization, the sections were washed twice in 2 \times SSC, once in 0.2 \times SSC and

twice in 0.1 \times SSC for 15 min per wash at 42°C. Then at room temperature, the sections were washed three times with PBS for 2 min per wash and then quenched by submerging into a methanol hydrogen peroxide solution (180 ml methanol in 20 ml 30% hydrogen peroxide). The tissue sections were further washed three times in PBS for 2 min per wash. Blocking and detection was performed using the Spot-Light CISH detection system (Zymed, San Francisco, CA, USA) according to the manufacturer's specification. Briefly, 100 μl of nonspecific blocking solution supplied with the kit (CAS-BlockTM) was added to the sections for 5 min. The sections were then incubated with FITC-labeled sheep anti-digoxigenin antibody followed by HRP-goat anti-FITC antibody. The supplied DAB chromagen was used for detection. The sections were then counter stained with Meyer's hematoxylin for 2 min and examined under light microscopy.

Statistics

All data are presented as mean \pm SE. Comparisons between groups used an unpaired, two-tailed *t*-test. Values of $p < 0.05$ were considered significant.

Results

Histology and immunocytochemistry

After three days, there was no intima evident in balloon-injured arteries. In stented arteries, a layer of thrombus and cellular material surrounding the stent struts constituted the nascent neointima. Adherent leukocytes on the luminal surface of both granulocyte and mononuclear morphology were evident in both injury groups, but were more numerous in stented arteries (17 ± 7 versus 232 ± 35 cells/section, $p < 0.001$). Immunocytochemical examination at three days (Figure 1) revealed infiltrative neutrophils

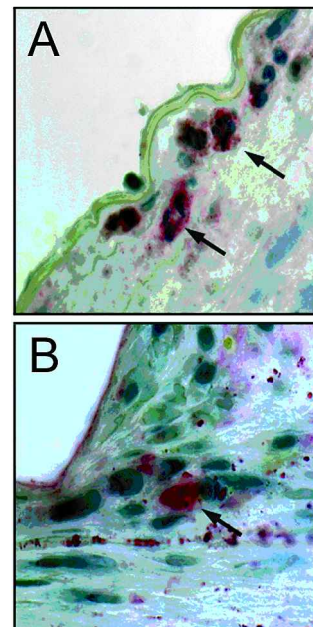


Figure 1 Photomicrograph (60 \times) shows immunostaining for inflammatory cell types in rabbit iliac arteries. (A) staining for neutrophils in rabbit iliac arteries (RPN3/57, three days post balloon injury). (B) staining for macrophages in rabbit iliac arteries (RAM-11, 14 days post stent).

within the media of balloon-injured arteries, but substantially more abundant cells within the neointima and media of stented arteries (37 ± 14 versus 95 ± 13 cells/section, $p < 0.03$, Figure 2).

After seven days a cellular neointima was evident in both balloon-injured and stented arteries. Adherent leukocytes of both neutrophil and mononuclear type remained more abundant in stented than in balloon-injured specimens (62 ± 11 versus 20 ± 9 leukocytes/section, $p < 0.01$). Fewer neutrophils than those identified within the media at day three were evident within the neointima after seven days in both groups (6 ± 4 cells/section in balloon injury versus 33 ± 6 cells/section in stent injury, $p < 0.03$, Figure 2). No leukocytes were identified within the media at seven days.

By 14 days, adherent leukocytes of both neutrophil and mononuclear type were still identifiable on the lumen of stented arteries, but virtually none were seen in balloon-injured arteries (52 ± 14 versus 2 ± 1 leukocytes/section, $p < 0.001$). Neutrophils were scarce at 14 days within the neointima of balloon-injured and stented arteries (1 ± 1 versus 7 ± 2 cells/section, $p = \text{NS}$, Figure 2) and none were seen within the media.

Monocytes were absent at all time points in balloon-injured arteries, but were present within the neointima of stented arteries progressively accumulating at later time points (24 ± 8 , 59 ± 23 , and 88 ± 12 cells/section at three, seven, and 14 days, respectively, Figures 1 and 2). None were identified within the media at any time point. Therefore, the inflammatory response in balloon-injured arteries was limited to early (three days) neutrophil infiltration. In contrast, in stented arteries, an early infiltration of neutrophils was followed by chronic infiltration of monocytes with persistent presence of adherent leukocytes as late as 14 days.

Semi quantitative reverse transcriptase PCR

In order to characterize signals that could be driving differential recruitment of inflammatory cells to the site of vascular injury, PCR analysis of stented and balloon-injured arteries was performed. After balloon injury, MCP-1 mRNA levels peaked at 4 h, returned to baseline at 8 h,

and were not detectable at 14 days (Figure 3). IL-8 levels followed a similar pattern, peaking at 8 h and not detectable at 14 days (Figure 3). In contrast, after stent-induced injury, there was a marked increase in MCP-1 and IL-8 mRNA levels hours after injury and message was still detectable at 14 days.

In situ hybridization

To further characterize the location of synthesis of these cytokines In situ hybridization for MCP-1 mRNA was per-

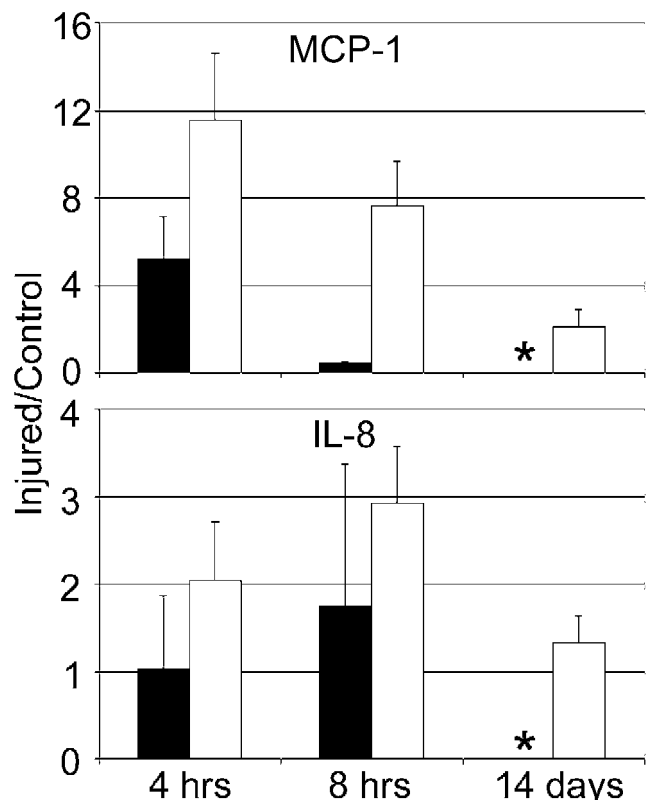


Figure 3 MCP-1 mRNA levels (injured/control) and IL-8 mRNA levels (injured/control) in balloon-injured vessels (solid bars) and stented vessels (open bars). *Undetectable levels.

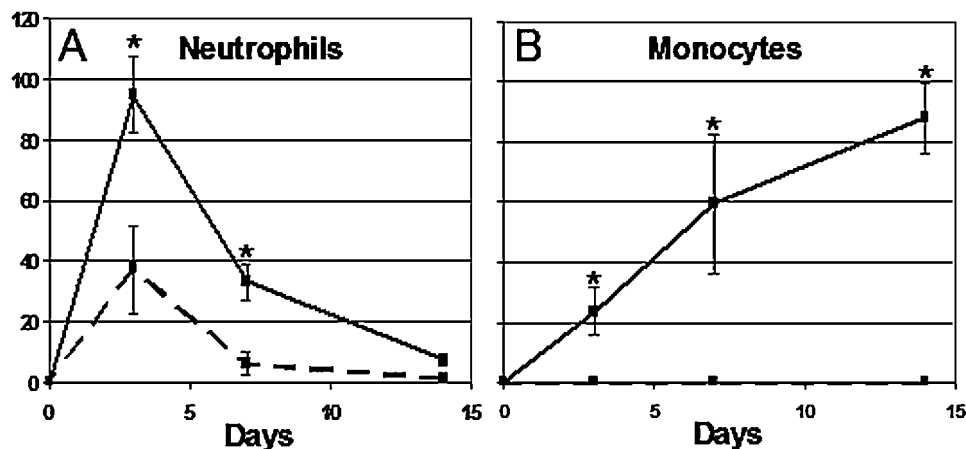


Figure 2 Line graph showing number of tissue leukocytes per arterial section as identified by immunostaining in stented arteries (solid line) and balloon-injured arteries (dashed line). * $p < 0.03$.

formed, revealing the presence of MCP-1 mRNA within the media at 4 h after stent implantation (Figure 4). At 14 days post stent implantation, MCP-1 mRNA can still be identified within the media.

Discussion

Leukocytes have long been known to play an important causative role in the development of human atherosclerosis.⁹ In addition, there is now abundant evidence to suggest that both monocytes and neutrophils play a role in restenosis following percutaneous intervention and in experimentally induced arteriopathies.^{10–16} Our data demonstrate that distinct patterns of leukocyte infiltration exist distinguishing the superficial injury associated with simple balloon-induced de-endothelialization from the deep chronic injury associated with stent implantation. Balloon injury is associated with early and transient infiltration of neutrophils without monocyte accumulation, while stent implantation is associated with an early influx of neutrophils followed by sustained recruitment of monocytes over days to weeks. These patterns of leukocyte accumulation are mirrored by chemokine mRNA levels. Following balloon injury, peak levels of mRNA for MCP-1 and IL-8 occur within hours but mRNA for these chemokines cannot be detected at 14 days post injury. In contrast, there is sustained presence of mRNA for MCP-1 and IL-8 for at least 14 days after stent implantation.

Leukocyte infiltration following vascular injury

Heparin, the archetypal modulator of vascular repair in animal models, is equally effective at reducing neointimal hyperplasia following balloon injury or stent implantation in a rabbit iliac artery model. However, our laboratory has shown that maximal inhibition of neointimal hyperplasia in stented arteries requires continuous heparin administration for the duration of the experiment, while transient early heparin therapy following balloon injury is just as effective as chronic administration.¹⁷ Our laboratory has also demonstrated that, in stented arteries, there is abundant recruit-

ment of macrophages within the neointima, and inhibition of macrophage accumulation corresponds tightly with reductions in neointimal growth.⁸ In balloon-injured rabbit iliac arteries, a model devoid of macrophage infiltration, neutrophil infiltration has been documented within hours of injury. Inhibition of neutrophil infiltration correlates with an inhibition of medial smooth muscle cell proliferation.¹⁶ In addition, we have demonstrated in a primate iliac artery model that monocyte-specific blockade achieved via blockade of the MCP-1 receptor CCR2, is effective at reducing neointimal hyperplasia after stenting.¹⁸ In contrast, in the same model, combined neutrophil and monocyte blockade achieved by targeting the leukocyte β_2 -integrin β -subunit CD18, is required to reduce neointimal hyperplasia after balloon injury. These data suggest that leukocyte infiltration is causally related to neointimal hyperplasia following balloon- or stent-induced injury, but that temporal and spatial patterns of leukocyte infiltration vary with different forms of arterial injury, unveiling different mechanistic targets for modulating cellular elements of repair. The current study confirms these hypotheses, showing distinct patterns of leukocyte infiltration in balloon-injured versus stented arteries. In addition, the current study adds to the mechanistic understanding of these processes by demonstrating distinct patterns of chemokine presence between the different forms of vascular injury.

Role of MCP-1 in vascular injury

Cipollone et al¹⁹ have demonstrated upregulated levels of MCP-1 following balloon angioplasty in humans and found that MCP-1 levels correlate with risk for restenosis. Similarly, several studies have looked at the role of MCP-1 after vascular injury in animal models. Taubman et al²⁰ demonstrated peak presence of MCP-1 mRNA following balloon injury in a rat carotid model at 4 h. Furukawa et al²¹ have recently reported an inhibitory effect of a polyclonal goat IgG against MCP-1 in a rat carotid injury model. Gu et al²² demonstrated significant reductions in lipid and monocyte accumulation in the aortas of mice genetically deficient in MCP-1 and the LDL receptor. Mice genetically deficient in CCR2 and apoE also demonstrate less extensive atheros-

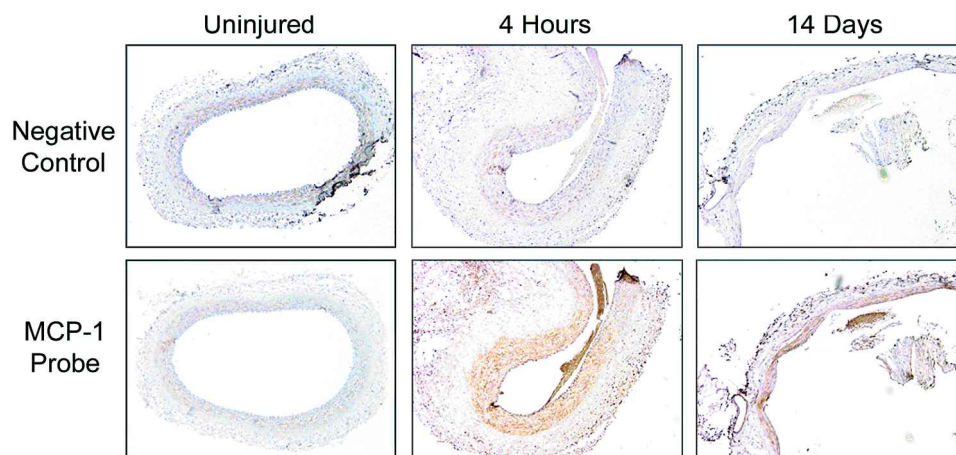


Figure 4 Photomicrographs (20 \times) showing results of in situ hybridization for MCP-1 mRNA. Upper row shows sections stained with negative control probe while bottom row shows staining with MCP-1-specific probe. Sections from uninjured arteries show no MCP-1 staining. At 4 h after stent placement, positive staining is seen within the media. At 14 days, positive staining is seen within the media. The neointima is stripped from the specimen and therefore not assessed.

clerotic lesion development. Recently, Ni et al²³ have demonstrated efficacy of anti-MCP-1 gene therapy against atherosclerotic lesion development in an apoE knockout mouse. The findings of this study confirm the earlier findings of Taubman et al,²⁰ showing peak levels of mRNA for MCP-1 within hours of balloon injury. We now demonstrate that in contrast to balloon-injured specimens, there is sustained presence of mRNA for MCP-1 mRNA as long as 14 days post stent implantation.

Role of IL-8 in vascular injury

IL-8 is the most important chemokine in modulating recruitment of neutrophils to sites of injury² and has been identified in human atherosclerotic plaque.²⁴ Interestingly, Gerszten et al²⁵ found in an in vitro model, that IL-8 also participates in triggering firm monocyte adhesion to cultured endothelial cells. In addition, in vitro experiments have shown that fibrin, prominent at sites of vascular injury, strongly promotes IL-8 mRNA expression from endothelial cells.²⁶ Hancock et al²⁷ confirmed measurable levels of IL-8 within rat carotid arteries 14 days after balloon injury. In human subjects undergoing angioplasty, Cipollone et al¹⁹ measured elevated levels of circulating IL-8, peaking at five days post procedure, although they were not able to correlate levels with the propensity for restenosis. We now report peak mRNA levels of IL-8 mRNA 8 h post both balloon- and stent-induced injury in the rabbit iliac artery model with sustained mRNA levels seen in stented arteries as long as 14 days post injury.

Limitations

Our study cannot eliminate the possibility that differences in temporal patterns of IL-8 and MCP-1 mRNA content between balloon-injured and stented arteries are a product of altered kinetics of mRNA degradation rather than differences in mRNA expression. Of particular relevance to the inflammatory response following vascular injury is hyperlipidemia. Elevated lipid levels, particularly of low-density lipoprotein, profoundly influence the number and type of leukocytes recruited, and play a well-recognized role in human atherosclerosis. For these reasons, it will be important to extend observations from the current study to hyperlipidemic animal models.

Conclusions

The present study builds upon evidence that there are important differences in the temporal and cellular characteristics of the inflammatory response to vascular injury following simple balloon denudation or implantation of a chronic indwelling stent. The inflammatory response after balloon injury is transient, consisting predominantly of neutrophils. In contrast, the inflammatory response following stent implantation is more prolonged and involves an early phase of neutrophil infiltration followed by accumulation of macrophages. Expression of the chemokines MCP-1 and IL-8 mirrors these cellular responses with transient expression of chemokines following balloon injury and sustained expression following stent implantation. These findings may be of critical importance in the choice and duration of anti-inflammatory therapies following percutaneous coronary intervention.

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