Journal of Surgical Research **160,** 9–13 (2010)
doi:10.1016/j.jss.2009.04.003

ASSOCIATION FOR ACADEMIC SURGERY

Serum Levels of Matrix Metalloproteinase-2 as a Marker of Intimal Hyperplasia

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Submitted for publication January 9, 2009

Background. **A primary component in the development of intimal hyperplasia (IH) in response to vascular injury is basement membrane remodeling. Matrix metalloproteinases (MMPs) play a major role in this process by degradation of basement membrane proteins, mainly collagen type IV. Vascular injury initiates an inflammatory cascade with the release of tumor necrosis factor-alpha (TNFα), interleukin-1beta (IL-1β), and C-reactive protein (CRP). We hypothesize serum levels of these elements may serve as biomarkers of the development of IH.**

Methods and Results. **At baseline, 2, 7, 10, and 14 days post-balloon angioplasty of the carotid artery, rat tissue samples were stained with Masson trichrome elastin to examine IH. Intima:media ratios (I:M) increased significantly over time postinjury. Serum samples were collected at the time of tissue sampling, and levels of MMP-2, MMP-9, collagen type IV, TNFα, IL-1β, and CRP were assayed using sandwich enzyme-linked immunosorbent assay (ELISA). MMP-2 serum levels at 7, 10, and 14 days postinjury were significantly elevated compared with baseline. Other elements were not significantly elevated.**

Conclusion. **Early and persistent elevation in the serum levels of MMP-2 may be a useful biomarker of basement membrane remodeling and the presence of IH.**

Key Words: **MMPs; intimal hyperplasia; biomarkers.**

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INTRODUCTION

Intimal hyperplasia (IH) is the general pathologic response of a vessel to injury and a common indication for secondary intervention in peripheral vascular disease. There are four major components of IH pathogenesis: extracellular matrix (ECM) degradation, vascular smooth muscle cell (VSMC) migration, VSMC proliferation, and excess ECM deposition. Migration of medial VSMCs to the intima region plays a major role in intimal wall thickening during vascular remodeling, seen in arterial disorders, such as atherosclerosis and restenosis following balloon angioplasty [1, 2]. Vascular remodeling at physiologic levels allows adaptation and repair after disruption of a vessel. However, pathologic remodeling underlies a major source of vascular intervention complications, and usually leads to the need for secondary intervention [3].

The inflammatory cascade is one of the primary response mechanisms to injury and infection. Vascular injury initiates an inflammatory reaction that contributes to vascular pathogenesis [4]. Inflammatory cytokines, such as interleukin-1 (IL-1), C-reactive protein (CRP), and tumor necrosis factor (TNF) have been shown to play specific roles [5, 6]. Analyses of these cytokines in IH development may prove beneficial in determining key regulatory factors or indicators of vascular restenosis.

The ability of VSMCs to migrate from the medial vascular layer to the intimal region is largely dependent on the degradation of their surrounding ECM. This degradation is primarily mediated by matrix metalloproteinases (MMPs), a family of extracellular endopeptidases [7, 8], and unbalanced MMP enzymatic activity is associated with pathologic vascular

0022-4804/09 $36.00

remodeling [3, 9–11]. MMP-2 and -9 play major roles in vascular remodeling due to their primary affinity for collagen type IV and laminin, the key components of the basement membrane separating the intimal and medial layers of the vessel wall. MMP expression can be induced by cytokines, growth factors, stress, or inflammation [12, 13]. Cytokines such as IL-1β and TNFα have been shown to up-regulate MMPs in a number of cell types, including fibroblasts, endothelial cells, and VSMCs [14–16].

This study was undertaken in order to investigate the potential for inflammatory cytokine and/or MMP levels as biomarkers of IH. In current practice, the only definitive way to determine the degree of IH is through vessel imaging or direct sampling of intraoperative specimens, invasive procedures with associated morbidity. We hypothesize that serum levels of inflammatory cytokines, collagen type IV breakdown fragments, and/or MMPs will predict the presence and/or degree of IH development in the postoperative period.

MATERIALS AND METHODS

Experimental Groups

Vascular disease preferentially affects men and postmenopausal women. To focus our attention in the female population, female Sprague-Dawley rats known to be past litter-bearing capability were used at 12 mo of age to best represent a well-defined patient population known to be affected by vascular pathology. After 1 wk acclimation, each rat was randomly assigned to an experimental group to be assayed at baseline, 2, 7, 10, or 14 days postinjury. All animal procedures conformed to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication no. 85-23, revised 1996), and were approved by the Institutional Animal Care and Use Committee, certified by the American Association of Accreditation of Laboratory Animal Care.

Balloon Angioplasty of the Carotid Artery

A standardized and accepted rat carotid artery balloon injury procedure was used to induce vascular injury to the left common carotid. Rats were anesthetized with vaporized 1%–5% isofluorane. A midline neck incision exposed the left carotid artery. A 2F balloon catheter was passed through a small arteriotomy in the external carotid artery, into the common carotid artery, and down to the aortic arch. The balloon was distended to two atmospheres of pressure and passed anti-grade and retro-grade through the common carotid to denude the endothelium. This was repeated three times, after which the catheter was removed, the external carotid ligated, and the incision closed. The right carotid artery was exposed by partial dissection, and served as the operational sham.

Carotid Artery Tissue Procurement

At baseline, 2, 7, 10, or 14 days postinjury, each animal was euthanized with an overdose of CO_2 gas, and the thoracic cavity was opened for full exposure. Vascular pressure perfusion and fixation was performed by making a small incision into the right atrium for fluid draining and inserting a 16-gauge needle into the left ventricle, attached to a saline bag pressure controlled by gravity. After 10 min of saline perfusion, the bag was switched to fresh 10% formalin for 10 min for tissue fixation, and the carotid arteries were extracted by fine microdissection.

Immunohistochemistry

The carotid arteries were placed in 10% formalin solution for 24 h for further fixation, paraffin embedded, cross sectioned transversely at 4 μm, and prepared for immunohistochemical analysis. Tissue sections were stained with hematoxylin and eosin to confirm denudation of the endothelial lining and the presence of basement membrane injury. Tissue sections were stained with Masson's trichrome elastin, according to standard operating procedures. Stained images were acquired using a BX51 Olympus microscope with an Olympus Q-color camera (Melville, NY) and quantified using Image ProPlus image analysis software (Media Cybernetics, Bethesda, MD). Intima to media ratios (I:M) were used as a quantification of IH development.

Serum Collection and ELISA Immunoassay

At the time of sacrifice 1 mL whole blood was collected, centrifuged at 1500 g for 5 min, serum aliquoted, and frozen at –80 °C until analysis. Serum levels of inflammatory cytokines and MMPs were measured by sandwich enzyme-linked immunosorbent assay (ELISA) to document their correlation to the degree of IH development over time. Rat-specific ELISA kits were purchased commercially for MMP-2, IL-1β (Quantikine Inc., Minneapolis, MN), TNF-α (Endogen, Inc., Woburn, MA), CRP (Chemicon, Inc., Billerica, MA), MMP-9, and collagen type IV (USCN-Life, Inc., Wuhan, China) and used as per the manufacturers' instructions.

Statistical Analysis

All data are reported as mean ± SEM. Statistical analyses were performed using Student's t-test or one-way ANOVA and a post-hoc Tukey's test using SigmaStat ver. 3.5 software (Systat, Chicago, IL). Bivariate correlation was used to analyze the relationship between variables. $P < 0.05$ was considered to be significant.

RESULTS

Intima to Media Ratio Increases Significantly Over Time

IH development was assayed at various time points post-balloon angioplasty, and I:M ratios were measured to quantify degree of IH development in response to injury over time. No measurable intima layer was present in baseline samples (I:M = 0, $n = 3$, Fig. 1A and B). I:M was elevated by the first postinjury measurement at day 2 ($0.15 \pm 0.04^{\#}$) and increased significantly by 10 and 14 days postinjury (7 days, 0.26 ± 0.04^{NS}; 10 days, $0.66 \pm 0.12^{\#}$; 14 days, $1.09 \pm 0.09^{\#}$; $^{\#}P < 0.05$ *versus* previous time point; $n = 3$, Fig. 1B).

MMP-2 Serum Levels Significantly Increase by 7 Days Postinjury and Persist Over Time

We have previously shown MMP-2 plays a significant role in VSMC migration, a major process in IH development [17]. MMP-2 serum levels were detected by sandwich ELISA as described previously. MMP-2 serum levels at 2 days postinjury (107 ± 18 ng/mL) were not significantly elevated over baseline levels of non-