Cardiovascular Pathology 25 (2016) 284–292

Contents lists available at ScienceDirect

Cardiovascular Pathology

Original Article

Role of macrophage colony-stimulating factor in the development of neointimal thickening following arterial injury

Vivek Mishra [a], Satyesh K. Sinha [b], Tripathi B. Rajavashisth [a,b,c,*]

[a] Molecular Biology Unit, Institute of Medical Sciences, Banaras Hindu University, Varanasi 221005, India
[b] Division of Endocrinology, Metabolism, and Molecular Medicine, Charles R. Drew University of Medicine and Science, Los Angeles, CA 90059, USA
[c] David Geffen School of Medicine at University of California Los Angeles, Los Angeles, CA 90095, USA

ARTICLE INFO

Article history:
Received 10 November 2015
Received in revised form 3 April 2016
Accepted 6 April 2016

Keywords:
Balloon injury
M-CSF
c-fms
Carotid artery
Neointimal proliferation
Smooth muscle cells

ABSTRACT

Evidence suggests that macrophage colony-stimulating factor (M-CSF) participates critically in atherosclerosis; little is known about the role of M-CSF in the development of neointimal hyperplasia following mechanical vascular injury. We examined the expression of M-CSF and its receptor, c-fms, in rodent and rabbit models of arterial injury. Injured rat carotid arteries expressed 3- to 10-fold higher levels of M-CSF and c-fms mRNA and protein following balloon injury as compared to uninjured arteries. In the rabbit, M-CSF protein expression was greatest in neointimal smooth muscle cells (SMCs) postinjury, with some expression in medial SMCs. M-CSF-positive SMCs exhibited markers of proliferation. At 30 days postinjury, neointimal SMCs in the adjacent healed area near the border between injured and uninjured zone lost both proliferative activity and overexpression of M-CSF. The presence of induced M-CSF and c-fms expression correlated with the initiation of SMCs proliferation. M-CSF stimulated incorporation of [^{3}H] thymidine in human aortic smooth muscle cells in a concentration-dependent manner. Serum-free conditioned medium from aortic SMCs also promoted DNA synthesis, and this effect was blocked by M-CSF specific antibody. To test further the role of M-CSF in vivo, we induced arterial injury by placing a periadventitial collar around the carotid arteries in compound mutant mice lacking apolipoprotein apoE (apoE$^{-/-}$) and M-CSF. Loss of M-CSF abolished the neointimal hyperplastic response to arterial injury in apoE$^{-/-}$ mice. Local delivery of M-CSF to the injured artery restored neointimal proliferation, suggesting a critical role of M-CSF for the development of neointimal thickening following arterial injury.

1. Introduction

Vascular smooth muscle cells (SMCs) normally localize primarily in the media of the normal artery with few or none in the intima, but during the development of atherosclerotic plaque, activated SMCs migrate from the media to the intima of the arterial wall [1–5]. Intimal SMCs may then proliferate, produce extracellular matrix macromolecules, form foam cells by taking up lipoproteins including modified low density lipoprotein (LDL) [2–5], and thereby contribute to the development of complex atherosclerotic lesions. The initiating event in this process is thought to be some form of insult to the artery that then induces a complex inflammatory response that ultimately goes awry, frequently culminating in devastating clinical events such as myocardial infarction and stroke [1,4].

Mechanical injury to the artery such as balloon angioplasty or other arterial interventions causes an analogous, albeit accelerated, process of atherosclerosis. While there are differences in these two pathologic responses to injury, there are also key similarities. For example, endothelial denudation of the arterial wall by a balloon or catheter leads to SMCs migration from media to intima followed by proliferation of intimal SMCs [3,5–10]. During this process, SMCs undergo phenotypic modulation, losing contractile proteins and augmenting production of extracellular matrix [6–11]. The resultant elaboration of matrix contributes importantly to the mass of the plaque and may eventually lead to loss of lumen and attendant poor clinical outcomes. The precise mechanisms instigating this SMCs phenotypic alteration are not well understood but are thought to involve various cytokines and growth factors within the microenvironment [1–4,10–12]; one such cytokine may be the macrophage colony-stimulating factor (M-CSF).

M-CSF is a multifunctional protein that regulates the differentiation, proliferation, and survival of mononuclear phagocytes by binding to a specific cell surface receptor encoded by the protooncogene c-fms [13–17]. Several lines of evidence suggest that M-CSF plays important roles in atherogenesis and the arterial response to injury: (1) besides

Funding: This work was supported by grants from the National Heart, Lung, and Blood Institute (HL51980 and HL58555 to Dr. Rajavashisth). VM was supported by a fellowship from the Council of Scientific and Industrial Research, India. SKS was supported by National Institutes of Health grants (HL30568 and U54-MD-008149). TBR was supported by a Ramalingaswami Fellowship from the Department of Biotechnology, Government of India.
* Corresponding author. Molecular Biology Unit, Institute of Medical Sciences, Banaras Hindu University, Varanasi 221005, U.P., India.
E-mail addresses: rajavashisth@ucla.edu, rajavashisth@gmail.com (T.B. Rajavashisth).

http://dx.doi.org/10.1016/j.carpath.2016.04.003
1054-8807/

its role in regulating growth and differentiation of mononuclear phagocytes, M-CSF also functions as a growth factor for other cells, including vascular SMCs [17–21]; (2) atherosclerotic lesions derived from humans and rabbits contain elevated levels of M-CSF mRNA and immunoreactive protein [22–24]; (3) all major cell types in vascular lesions — endothelial cells (ECs), SMCs, and monocyte macrophages — can express M-CSF [22–25]; (4) oxidized LDLs, known to play a pivotal role in the pathobiology of atherosclerosis [1–4], induce the expression of M-CSF in aortic ECs and SMCs [22,25]; (5) absence of M-CSF in atherosclerotic apoE or LDL receptor knockout mice prevents plaque development despite augmented hypercholesterolemia [26–29]; and (6) intimal SMCs isolated from an experimental rabbit model of atherosclerosis express c-fms and proliferate in response to M-CSF [18,19]. However, while these data are intriguing and suggestive, direct evidence implicating M-CSF in the pathogenesis of neointimal thickening following arterial injury has, heretofore, been lacking.

We hypothesized that increased vascular M-CSF expression is an early response to arterial injury and is required for SMCs proliferation and neointima formation. Using three animal models, we report here that arterial injury leads to a rapid induction in the expression of both M-CSF and c-fms mRNA and protein and that these events immediately precede SMCs proliferation. We further demonstrate that, in mice bearing a mutation rendering them unable to express M-CSF, the neointimal response to arterial injury is abolished, but it can be restored by local delivery of exogenous M-CSF to the injured artery. Moreover, we show that human aortic smooth muscle cells (HASMCs) in tissue culture express c-fms and proliferate in response to exogenously added M-CSF. Our results directly implicate and, for the first time, demonstrate a critical role for M-CSF signaling in altering SMC properties that result in neointima formation after arterial injury.

2. Materials and methods

2.1. Animals

This study utilized three species of animals: (1) male Sprague–Dawley rats ($n=5$); (2) New Zealand white rabbits ($n=18$); and (3) apoE knockout mice ($n=5$) with or without M-CSF deficiency. Rats were purchased from Tyler Labs (Bellevue, WA) and, at the time of carotid artery injury, were 3–4 months old and weighed 400 g. Rabbits were purchased from Millbrooks Farms (Amherst, MA) that weighed approximately 3 kg at the time of surgery. Breeding pairs of homozygous apoE knockout mice on a C57BL/6J background [30] and heterozygous (*op*/+) mice on a C57BL/6JxC3HeB/FeJ hybrid genetic background [31,32] were purchased from the Jackson Laboratory (Bar Harbor, Maine). Heterozygous (*op*/+) mice were subsequently backcrossed for six generations to inbred strain C57BL/6J mice to produce a homogeneous genetic background. Mice lacking both apoE and M-CSF were produced by crossing apoE knockout mice with *op*/+ mice essentially as described [27,28]. ApoE-null mice lacking M-CSF due to a point mutation in the M-CSF gene showed impaired growth and differentiation of monocytes and their precursors in bone marrow, causing a deficiency of blood monocytes and peritoneal and tissue macrophages [27,28]. These mice also displayed the osteopetrotic phenotype due to severe deficiency of osteoclasts, resulting in impaired bone remodeling and skeletal deformities [27,31,32]. Mice were fed a high-fat, high-cholesterol atherogenic diet containing 42% (wt/wt) fat and 0.15% cholesterol (Harlan Teklad, CA) from 6 weeks of age (18–20 g) through the duration of the experiment. Animal husbandry of toothless (*op*/*op*) mice was performed using a liquid suspension of a powdered diet to maintain the body weight as described previously [27,28]. Genotyping and animal husbandry of the mutant were performed according to our published procedure [27,28]. Experimental protocols involving these animals were approved by the Institutional Animal Care and Use Committee.

2.2. Cultured cells

The isolation and characterization of HASMCs from aortae of human heart donors has been previously described [33]. HASMCs were cultured in M199 medium (GIBCO BRL, Grand Island, NY) containing 20% fetal calf serum (FCS), penicillin (100 U/ml), streptomycin (100 μg/ml), and 25 mM Hepes (pH 7.4). Cells from passages 5–7 were used. Peripheral blood monocytes were isolated as described previously [34]. Monocyte-derived Mϕ were cultured in RPMI 1640 containing 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B for 7 days. All reagents in our tissue culture studies were verified for the absence of endotoxin by a commercially available assay kit (BioWhittaker, Walkersville, MD) that has a sensitivity detection level of 1 pg/ml.

2.3. Rat carotid artery injury model

Rats ($n=5$) were maintained on a normal rat chow diet. At age 3–4 months, these rats were anesthetized and the left common carotid artery was injured with a balloon catheter as previously described [8,35]. At 0, 6, 24, and 48 h; 3, 4, and 5 days; and 1, 2 and 3 weeks following injury, the animals were euthanized and both injured (left) and uninjured (right) common carotid arteries were retrieved for Northern and Western blot analyses of M-CSF and c-fms expression. The arteries were stripped of periadventitial fatty and connective tissues in phosphate-buffered saline (PBS) at 4°C. Endothelium of the right carotid artery was removed by gently scraping the luminal surface with the edge of a Teflon card. Efficacy of this procedure was verified by loss of the hybridization signal for von Willebrand factor (vWf) mRNA. Harvesting and preparation of tissues for RNA and protein analyses were essentially as previously described [35].

For Northern blotting, total cellular RNA from frozen arterial tissues ground to a fine powder under liquid nitrogen was isolated by lysis in acid guanidinium isothiocyanate, phenol–chloroform extraction and ethanol precipitation as previously described [36]. Each RNA preparation (15 μg) was denatured and electrophoresed through a 1.2% formaldehyde agarose gel followed by blotting onto to nylon membranes and ultraviolet (UV) cross-linking. Ethidium bromide staining of 28S and 18S ribosomal RNA was used to verify equal loading in each lane. Filters were hybridized with isolated and radiolabeled M-CSF and c-fms specific cDNA probes [37,38]. The blots were washed and autoradiographed. Quantitative results of the assays were obtained by densitometry of autoradiograms.

For immunoblotting, extracts of frozen arterial tissues were isolated as described [35]. Proteins of arterial tissues (50 μg) and known molecular weight markers (Bio-Rad Laboratories, Hercules, CA) were separated by sodium dodecyl sulfate/polyacrylamide gel electrophoresis (10%, running, 4% stacking gel). Proteins were electrophoretically transferred onto Western polyvinylidene difluoride membranes and incubated overnight at 4°C with blocking solution (1% skimmed milk in PBS with 0.1% Tween 20). Affinity-purified rabbit polyclonal antibodies (10 μg IgG/ml) to mouse M-CSF or c-fms were incubated with the blots overnight at 4°C in PBS buffer containing 0.1% Tween 20. The blots were washed twice with PBS buffer and then treated with goat antirabbit antibody (1:5000 dilution) coupled to horseradish peroxidase. Immunodetection was accomplished using the Enhanced Chemiluminescence Kit (Amersham Inc.). Quantitative results of the assays were obtained by densitometry of autoradiograms.

2.4. Rabbit abdominal aortae injury model

This study used the aortic tissue samples generated by a previously reported rabbit abdominal aortae injury model by Tanaka et al. [39] for the localization of DNA synthesis and M-CSF expression by immunohistochemistry. Serial cryostat sections (6 μm) were cut, air dried onto poly-L-lysine coated slides, and fixed in acetone at −20°C for 5 min.