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Upregulation of Protein Kinase Cδ in Vascular Smooth Muscle Cells Promotes Inflammation in Abdominal Aortic Aneurysm

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Background. **The development of abdominal aortic aneurysms (AAAs) involves a complex interplay of extracellular matrix degradation, inflammation, and apoptosis. We have previously shown that protein kinase Cδ (PKCδ) plays a critical role in vascular smooth muscle cell (vSMC) apoptosis in the setting of oxidative stresses. Here, we show that PKCδ is also involved in the signaling that draws inflammatory cells to aneurismal tissue.**

Materials and methods. **Immunostaining for monocyte chemotactic factor (MCP)-1 and PKCδ was performed on paraffin-fixed arterial sections. Enzyme-linked immunosorbent assay to detect MCP-1 produced by vSMCs was performed on media from cultured rat A10 cells after cytokine induction with or without the PKCδ-specific inhibitor rottlerin. Migration of isolated lymphocytes was evaluated in response to media from activated A10 cells.**

Results. **Human AAAs show widespread and elevated expression of PKCδ that is not seen in normal aortic tissues. Cytokine stimulation of cultured vSMCs induced vigorous production of the key chemotactant MCP-1, the expression of which was PKCδ dependent. Stimulated vSMCs were capable of inducing the migration of leukocytes, and this effect was also dependent on PKCδ activity. Staining of human AAA tissue for MCP-1 showed an expression pattern that was identical to that of PKCδ and smooth muscle specific alpha-actin.**

Conclusions. **PKCδ is widely expressed in human AAA vessel walls and mediates MCP-1 expression by vSMCs, which could contribute to the inflammatory process. These findings, coupled with earlier studies of PKCδ, suggest that PKCδ plays a central role in the pathogenesis of AAAs and may be a potential target for future therapies.**

Key Words: **protein kinase Cδ; monocyte chemotactic factor-1; abdominal aortic aneurysms; aneurysm formation; vascular inflammation.**

INTRODUCTION

Abdominal aortic aneurysm (AAA) is a progressive and lethal disorder that is the 10th leading cause of death in men over the age of 55 in the United States [1]. The mainstay of treatment is either traditional open or endovascular surgical intervention and there is no medical bridging therapy that had proven to be effective to date [2, 3]. The development of this devastating disorder is a dynamic and complex process that involves an intricate interplay of matrix degradation, apoptosis, and inflammation [4–6].

Central to the development of AAA is a chronic inflammatory state where both resident vascular smooth muscle cells (vSMCs) as well as invading macrophages release matrix metalloproteinases, which gradually degrade critical extracellular matrix (ECM) components [7–10]. The cause of this chronic inflammatory state is incompletely understood but several stimuli have been implicated, including atherosclerosis, oxidative stress, angiotensin II, tumor necrosis factor alpha (TNFα), interleukin-1β, interleukin-6, and interferon-γ [11]. More recent work has shown that C-C motif chemokine receptor (CCR)-2, the receptor for monocyte chemotactic factor (MCP-1), is necessary to induce vascular inflammation. Inhibition of the MCP-1 signaling by means of CCR2 gene "knockout" blocks the recruitment of macrophages and thus the development of aneurysms in a mouse AAA model [12].

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The protein kinase C (PKC) family is a multimember group of cell membrane associated serine/threonine kinases. The kinases of the "novel" subfamily of PKCs, including PKCδ, are grouped together because their regulatory domain lacks calcium co-coordinating side-chain residues [13]. PKCδ has been linked to cell cycle control as well as cellular apoptosis in multiple cell types. It also has been shown that PKCδ can induce the expression of nuclear factor kappa B (NF-κB), the inhibition of which also directly suppressed AAA development in an animal model [14, 15]. NF-κB is a widely expressed transcription factor that is central in initiating and promoting an inflammatory response. Our own laboratory has shown that PKCδ is necessary for fibronectin synthesis as well as migration and proliferation of vSMCs, which are both central to maintaining the vessel wall matrix [16, 17].

To determine if a link existed between PKCδ and the development of AAAs, we screened a number of human samples for the expression of this and other proteins. Here we report the key role that PKCδ plays in induction of MCP-1 and the effect these proteins have on the migration of inflammatory cells. A greater understanding of the cellular mechanisms responsible for the chronic inflammation of AAAs is essential if pharmacological therapies are to be targeted at this devastating disease.

MATERIALS AND METHODS

General Materials

Rat TNFα was obtained from R&D Systems (Minneapolis, MN). Rottlerin was obtained from Calbiochem (San Diego, CA). Dulbecco's modified Eagle's medium and cell culture reagents were from Life Technologies, Inc. (Carlsbad, CA). MCP-1 antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Chemicals, if not specified, were purchased from Sigma Chemical Co. (St. Louis, MO).

Human Tissue Procurement

Eight human AAA specimens obtained from patients undergoing surgical repair of AAA and compared to two normal controls were obtained by members of the Pathology Department from autopsies performed on age-equivalent individuals that died from noncardiovascular diseases. No patients with known connective tissue disorders were included. The use of human samples in this study was approved by the Institutional Review Board at Weill Cornell Medical College.

Immunohistochemistry

Immunostaining for MCP-1 and PKCδ was performed on a paraffin-fixed arterial section of a diameter of 5–7 μm. MCP-1 antibody was used at 2 μg/mL and PKCδ at 1 μg/mL. A negative control was performed with each stain using IgG from the matching species and the same secondary antibody to ensure that no cross-reactivity or background staining occurred. Stained sections were digitally photographed.

Enzyme-Linked Immunosorbent Assay (ELISA) for MCP-1

ELISA to detect MCP-1 in smooth muscle cells (SMCs) was performed using rat MCP-1 ELISA kit (BD Biosciences, Bedford, MA). SMC were cultured at a density of 1×10^5/mL in 1 mL of complete medium in the presence or absence of different stimuli in 6-well Costar plates (Corning Inc., Corning, NY). After incubation for various periods of time at 37°C, cell-free culture supernatants were obtained. The concentrations of MCP-1 were then measured according to the manufacturer's instructions.

Cell Infection with Adenoviral Vectors

Adenoviral vectors expressing PKCδ were constructed as previously described [16]. Cells were plated at 80,000 cells per well in a six-well plate. Cells were exposed to 30,000 particles/cell for 4 h and then recovered in standard high-glucose Dulbecco's modified Eagle's medium overnight prior to treatment. Use of a green fluorescent protein-tagged adenovirus showed >80% infection and Western blot of cell lysate after exposure showed a strong band of expression consistent with PKCδ overexpression.

Isolation of Rat Bone Marrow

Bone marrow from Sprague Dawley rats was isolated by flushing the long bones with Dulbecco's phosphate-buffered saline containing 2% bovine serum albumin, and heparin (1000 U/mL). Cells from individual animals were pooled and suspended in the above medium and filtered through a 40-μm cell strainer (BD Biosciences). Viable lymphocytes were isolated from other blood components using Lympholyte-M (Cedarlane Laboratories Ltd., Hornby, Ontario) density centrifugation. The cells were then rinsed twice in phosphate-buffered saline followed by centrifugation.

Cell Migration Assay

Bone marrow derived lymphocytes (1×10^5) or 2×10^5 Raw 264 cells were placed in the upper chamber of Costar 24-well transwell plates with 5-μm pore filters (Corning Inc.) and the chamber was placed in a 24-well culture dish containing cultured conditional medium. After incubating plates for 6 h at 37°C, migrated cells were collected from the lower chambers and counted while remaining cells in the upper chamber were discarded. Cells were then stained with Calcein AM (Invitrogen, Eugene, OR) fluorescent nuclear stain and nucleated cells were counted.

Statistics

All data are expressed as means ± SEM. Continuous variables including MCP-1 concentrations and migration assay results were analyzed via paired Student's *t*-test. All statistical analysis was carried out using the statistical package on Microsoft Excel (Microsoft Corporation, Redmond, WA).

RESULTS

PKCδ Is Highly Expressed in the Aortic Wall of Human AAAs

The link between PKCδ and pro-inflammatory cytokines or oxidative stresses led us to evaluate a series of human abdominal aortic aneurysms obtained from surgical patients for the expression of this protein. AAA tissue samples were then compared to tissues obtained from autopsies of age-similar patients without aortic aneurysms. Compared to normal aortic wall, the AAA tissues have a disordered and diminished expression of smooth muscle-specific alpha-actin (αSMA)