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Shear Stress Influences the Release of Platelet Derived Growth Factor and Basic Fibroblast Growth Factor by Arterial Smooth Muscle Cells*

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Objectives: To determine the correlation between haemodynamic forces and the release of two mitogens for smooth muscle cells (SMC): Platelet Derived Growth Factor (PDGF) and basic Fibroblast Growth Factor (bFGF). Methodology: Bovine aortic smooth muscle cells were seeded on fibronectin coated polystyrene cylinders and allowed to reach confluence. The cells were subjected to a laminar flow of 50 cc/min (3 dyne/cm^2), 100 cc/min (6 dyne/cm^2) and 150 cc/min (9 dyne/cm^2) in an in vitro *system. Control cells were subjected to similar incubation conditions without flow. Principal results: Shear stress increased the release of mitogens by SMC. The release of mitogens was proportional to the level of shear stress and was still evident 24 hours after flow cessation. Conditioned serum-free medium from SMC subjected to shear stress increased tritiated thymidine uptake in Swiss 3T3 fibroblasts 13-fold as compared to conditioned serum-free medium from control SMC not subjected to shear stress ($p < 0.01$) and threefold as compared to standard control ($p < 0.001$). Addition of an excess of anti-PDGF antibody reduced the mitogenic activity of the conditioned medium by 30% ($p < 0.01$). Addition of an excess of anti-bFGF antibody reduced the mitogenic activity of the conditioned medium by 60% ($p < 0.01$). Conclusions: Increasing shear stress promotes the release of both PDGF and bFGF from arterial SMC in culture and is a possible explanation for atherosclerosis formation.*

Key Words: Shear stress; Smooth muscle cells; Platelet derived growth factor; Fibroblast growth factor; Atherosclerosis.

Introduction

The proliferation of arterial Smooth Muscle Cells (SMC) with subsequent intimal thickening is a key event in the development of atherosclerosis. Atherosclerosis localises preferentially in areas of flow disturbance and the predilection for atherosclerotic plaque to form at arterial bifurcations is well documented.[1,2] However the stimuli for the abnormal proliferation of SMC which characterises atherosclerosis are ill defined. Nor is it known why atherosclerosis localises preferentially in areas of flow disturbance. Two of the best characterised mitogens for SMC include Platelet Derived Growth Factor (PDGF) and basic Fibroblast Growth Factor (bFGF).

PDGF consists of glycoproteins that exist as dimer of two distinct but related chains termed PDGF-A and -B.[3,4] PDGF is a major mitogen from connective tissue cells in culture and has been shown to be chemotactic for SMC.[5] In normal adult SMC, PDGF is expressed at low concentrations but its expression is increased after tissue injury. Several reports have demonstrated increased expression of PDGF in association with naturally occurring atherosclerosis, experimentally induced atherosclerosis and with the myointimal hyperplasia associated with failure of vascular grafts.[6–8]

Basic Fibroblast Growth Factor (bFGF) is a member of the family of heparin binding mitogens, characterised by their affinity for heparin and their ability to stimulate both endothelial cell and SMC proliferation.[9] One striking feature of bFGF, revealed by DNA sequence analysis is the absence of a secretory signal peptide sequence, suggesting that the protein requires a chaperone to be secreted or is released after severe cell injury and/or cell lysis with secondary absorption on to the matrix.[10–12] Thus, SMCs can release PDGF and bFGF, thereby substaining their own growth. Any stimulus which increases PDGF

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and bFGF release by SMC could promote SMC proliferation and atherosclerosis formation. The hypothesis underlying our work is that abnormal haemodynamic conditions induce SMC proliferation by influencing the release of these mitogens.

The aim of our study was to analyse the correlation between shear stress and release of PDGF and bFGF by arterial SMCs in culture.

Material and Methods

Cell preparation and application of shear stress

SMCs were obtained from bovine thoracic arteries by collagenase digestion according to a method already described.[13] The cells were seeded in culture dishes (Falcon Plastics, Oxnard Ca) in a medium consisting of Dulbecco Modified Eagle Medium (DMEM) supplemented with 20% Fetal Calf Serum (FCS) and antibiotics and the cultures were kept at 37°C in an atmosphere of 5% CO_2 in air. Medium was changed after one day and thereafter every third day. SMCs were identified by positive staining with a monoclonal antibody to alpha actin (Fig. 1). SMC (5×10^5)

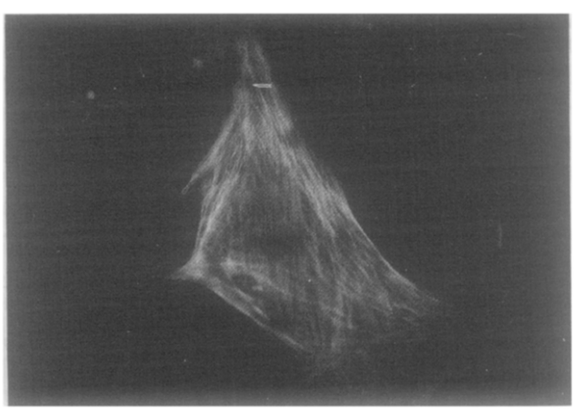

Fig. 1. Identification of bovine aortic smooth muscle cells by positive staining for alpha-actin (×500).

were seeded in fibronectin coated polystyrene tubes (Falcon Plastics, Oxnard Ca) and allowed to reach confluence and to adhere for 48 hours. Then the cylinders were interposed in a laminar flow system. The circulatory system consisted of a closed loop rolling pump. At this point the flow medium consisted only of DMEM without FCS, in order to accurately assess the release of bFGF and PDGF. SMC were subjected to different flow velocities. Three flow velocities weere chosen: 50 cc/min, 100 cc/min and 150 cc/min, which according to Poiseuille's law gave a shear stress of 3, 6 and 9 dyne/cm^2, respectively.

Assay for the presence of PDGF-like and bFGF molecules in the conditioned media

The presence of PDGF and bFGF molecules in the serum free conditioned media from SMC subjected to shear stress was determined by an inhibition antibody-binding assay. Dilutions of polyclonal rabbit anti-PDGF antibody (Genzyme Co, Boston MA, U.S.A.) or polyclonal mouse anti-bFGF antibody (4 μg/ml) were incubated with various dilutions of conditioned media in 400 micro tubes precoated with Phosphate Buffer Saline-Bovine Serum Albumin 1% (PBS-BSA 1%). After 20 hours of incubation at 4°C protein-A was added and the immunoaggregates were removed by centrifugation.

The residual antibody-binding activity in the supernatant was measured by Enzyme Linked Immunosorbent Assay (ELISA). In brief, plastic wells (96 wells, Falcon) were coated either with PDGF or bFGF (10 ng per well) for 8 hours at 4°C. Plates were then washed twice with PBS and saturated with PBS-BSA 1% for 2 hours at 37°C. Wells were then filled with 50 μl of supernatant obtained after immunoprecipitation. After 20 hours of incubation at 37°C the wells were washed with PBS-BSA 0.1%. Alkaline phosphatase labelled goat anti-rabbit Ig antibody was added. After incubation at 37°C the plates were washed three times in PBS-BSA 0.1% and once in distilled water. Finally *p*-nitro-phenylphosphate as substrate for the enzyme was added.

Bound specific antibody was quantitatively measured by optical density reading at 410 nm using a Perkin-Elmer Lambda spectrophotometer. We used anti-PDGF and anti-bFGF rabbit and mouse antibodies as positive control and rabbit and mouse IgG as negative control.

Assessment of release of mitogens

Serum-free conditioned media were collected from SMC subjected to the various levels of shear stress and from SMC not subjected to shear stress. Media were collected, centrifuged at 15 000 rpm, and stored at −20°C. Swiss 3T3 fibroblasts were used to assay the DNA synthesis-stimulating activity of the conditioned media. These cells bear receptors for PDGF and bFGF. Conditioned media (200 μl) or control media were added to subconfluent Swiss 3T3 cells. Positive controls received an equivalent volume of DMEM plus PDGF (Genzyme Co, Boston MA, U.S.A.) or bFGF (Genzyme Co, Boston MA, U.S.A.). Negative controls received serum-free DMEM.

Tritiated thymidine (1 microCurie per well) was added and the cultures were incubated for 18 hours and collected on glass fibre filters for radioactivity determination in an LKB scintillation counter.

Assay of reduction of mitogenic activity by anti-PDGF and anti-bFGF antibodies

Measurement of the ability of conditioned media from SMC subjected to shear stress to stimulate DNA synthesis of Swiss 3T3 cells was repeated in the presence of an excess of monospecific anti-PDGF and anti-bFGF.

Results

Presence of PDGF-like and bFGF molecules in the conditioned media

There were PDGF-like and bFGF molecules in the serum-free conditioned media obtained from SMC subjected to shear stress as shown by the shape of the antibody binding curves (Figs 2 and 3).

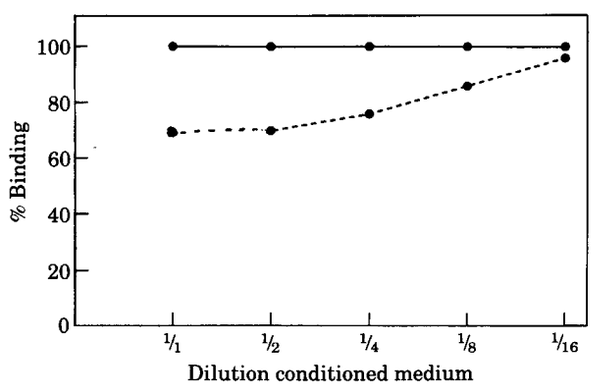

Fig. 2. Antibody-binding curve for PDGF-like molecules in the serum free medium from SMC subjected to shear stress (6 dyne/cm^2).

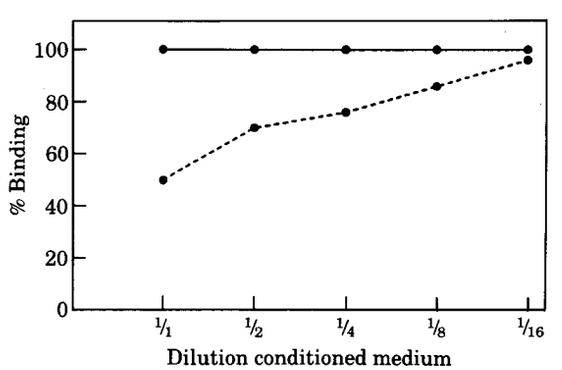

Fig. 3. Antibody-binding curve for bFGF in the serum free medium from SMC subjected to shear stress (6 dyne/cm^2).

Mitogenic activity in the conditioned media

Addition of conditioned medium (50% vol/vol) from SMC subjected to shear stress produced a mean increase of tritiated thymidine uptake of 13-fold as compared to negative controls and three-fold as compared to conditioned medium from SMC not exposed to flow in 3T3 cells (Fig. 4). This effect was proportional to the level of shear stress and it was still evident using conditioned media harvested 48 hours after flow cessation.

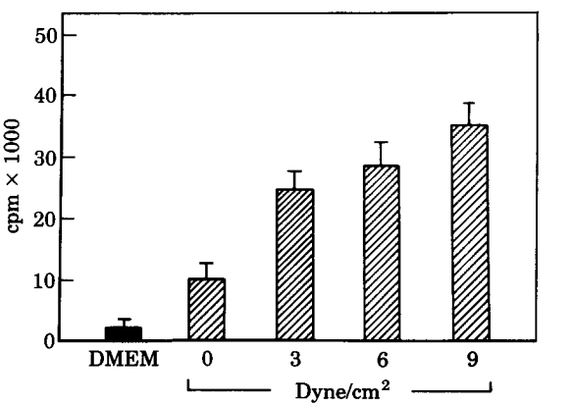

Fig. 4. Addition of 50% vol/vol conditioned medium from smooth muscle cells subjected to shear stress (6 dyne/cm^2) produced a mean increase of tritiated thymidine uptake of Swiss 3T3 cells of 13-fold as compared to negative controls and three-fold as compared to conditioned medium from SMC not exposed to flow.

Reduction of the mitogenic activity by anti-PDGF and anti-bFGF antibodies

Addition of anti-PDGF antibody to the medium of 3T3 cell cultures exposed to conditioned medium from SMC subjected to shear stress decreased the uptake of tritiated thymidine by 3T3 cells by 30% ($p < 0.1$) (Fig. 5).

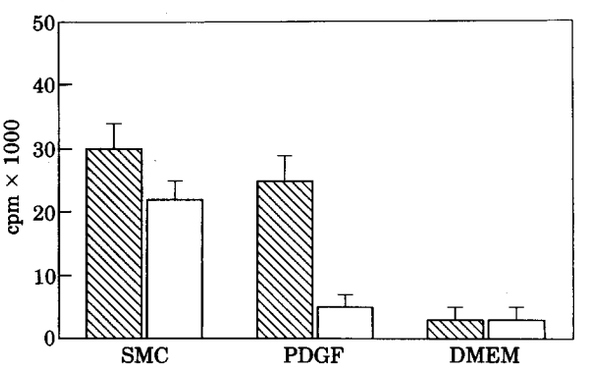

Fig. 5. Uptake of tritiated thymidine by Swiss 3T3 cells exposed to conditioned media from SMC subjected to shear stress. (□) with anti-PDGF antibody; (▨) without anti-PDGF. Addition of anti-PDGF antibody decreased the uptake of tritiated thymiline by 30%. DMEM negative control.

Addition of anti-bFGF antibody to the medium of 3T3 cell cultures exposed to conditioned medium from SMC subjected to shear stress decreased the uptake of tritiated thymidine by 3T3 cells by 50% ($p < 0.001$) (Fig. 6).

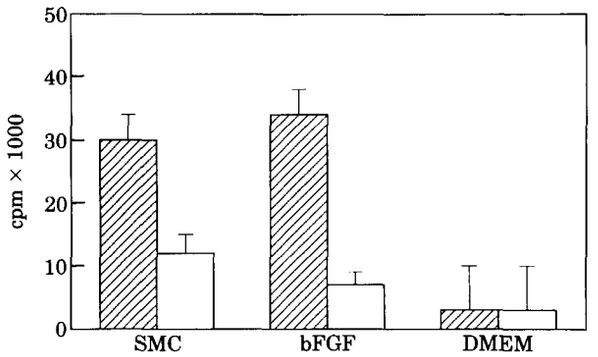

Fig. 6. Uptake of tritiated thymidine by Swiss 3T3 cells exposed to conditioned medium from SMC subjected to shear stress. (□) with anti-bFGF antibody; (▨) without anti-bFGF. Addition of anti-bFGF antibody decreased the uptake of tritiated thymidine by 50%. DMEM negative control.

Discussion

The predilection of the atherosclerotic plaque for the bifurcations of arteries or in segments with a sharp curvature is well documented.[1,2] Our findings support the hypothesis that haemodynamic factors are involved in the formation and progression of atherosclerosis. Previous studies have suggested a correlation between plaque formation and regions of flow separation and low shear stress.[14,15]

How haemodynamic factors influence the development of atherosclerosis and the proliferation of intimal SMC is unknown. It is known that arterial SMC in culture produce peptide growth factors which facilitate their own growth.[16,17] It is logical to consider that any stimulus which increases the release of these growth factors *in vivo* will promote SMC proliferation and therefore atherosclerosis formation. PDGF and bFGF are two well known mitogens for SMC. Our cell culture studies have shown that increasing shear stress promotes the release of PDGF and bFGF. *In vivo* increased shear stress also may predispose to atherosclerosis formation.

How increasing shear stress promotes the release of PDGF and bFGF needs clarification. The mechanisms probably involved are different for the two growth factors. Hsieh *et al.*[18] have shown that shear stress increases PDGF mRNA levels in endothelial cells. Resnick *et al.*[19] found that shear stress determined expression of PDGF-B in endothelial cells by inducing transcription factors interacting with a promoter element. But, which is the signal promoting increased production of PDGF? Shear stress induction of PDGF expression may depend on a signal transduction mechanism involving protein Kinase C activation. Previous work[20,21] has shown that shear stress stimulates phosphoinositide turnover in endothelial cells, producing the second messengers inositol trisphosphate and diacylglycerol which stimulate protein kinase C activity. The mechanism involved in bFGF release is probably different as bFGF lacks a secretory signal peptide sequence. However, it has been shown that severe injury and/or cell lysis results in release of bFGF.[10,11] Thus, increasing shear stress might promote bFGF release merely by injury to SMC.

It is evident that SMC can release growth factor and sustain their own growth. Increasing shear stress favours this phenomenon. But how do the findings of our study correlate with previous haemodynamic studies? Using models of the carotid bifurcation, Zarins *et al.*[14] and Lo Gerfo *et al.*[15] have found that the early atherosclerotic plaque localises in areas of flow stagnation and low shear stress. They concluded that low shear stress induces plaque formation. However, we know that atherosclerosis does not form in the venous system, where flow stagnation is common, or below an arterial occlusion where an area of low shear stress is generated. Therefore, we hypothesise that it is the simultaneous occurrence of high and low shear stress which favours atherosclerosis formation. Flow separation could provide a critical condition with areas of low shear stress where SMC are prone to proliferate and areas of high shear stress with SMC releasing high quantity of mitogens. These mitogens could influence directly the proliferation rate of the neighbouring SMC in areas of low shear stress.

Extension of these findings to the clinical setting raises the probability that any type of injury, be it mechanical or chemical, to the quiescent SMC might initiate release of bFGF to stimulate SMC proliferation. Experimental studies support this hypothesis. Administration of polyclonal antibodies to PDGF resulted in a 40.9% reduction in the area of neointima of myointimal lesions induced by intraarterial balloon catheter injury in athymic nude rats.[22] Similarly, Lindner and Reidy[23] have shown that proliferation of SMC after balloon injury of the rat carotid artery was inhibited by antibodies against bFGF. The proliferation of medial SMC measured 41 hours after balloon catheter injury, by tritiated thymidine uptake, was significantly reduced in those animals that received anti-bFGF antibody (1.5 *vs.* 7.6% in controls). A possible clinical application of these antibodies against

growth factors in humans is improbable. However, identification of the mechanisms involved in the release of PDGF and bFGF is of clinical significance. Only then can substances be developed that could block growth factor release and reduce the formation and progression of atherosclerosis.

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