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Research Article

Phenotypic heterogeneity influences the behavior of rat aortic smooth muscle cells in collagen lattice

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Abstract

Phenotypic modulation of vascular smooth muscle cells (SMCs) in atherosclerosis and restenosis involves responses to the surrounding microenvironment. SMCs obtained by enzymatic digestion from tunica media of newborn, young adult (YA) and old rats and from the thickened intima (TI) and underlying media of young adult rat aortas 15 days after ballooning were entrapped in floating populated collagen lattice (PCL). TI-SMCs elongated but were poor at PCL contraction and remodeling and expressed less α2 integrin compared to other SMCs that appeared more dendritic. During early phases of PCL contraction, SMCs showed a marked decrease in the expression of α-smooth muscle actin and myosin. SMCs other than TI-SMCs required 7 days to re-express α-smooth muscle actin and myosin. Only TI-SMCs in PCL were able to divide in 48 h, with a greater proportion in S and G2-M cell cycle phases compared to other SMCs. Anti-α2 integrin antibody markedly inhibited contraction but not proliferation in YA-SMC–PLCs; anti-α1 and anti-α2 integrin antibodies induced a similar slight inhibition in TI-SMC–PCLs. Finally, TI-SMCs rapidly migrated from PCL on plastic reacquiring their epithelioid phenotype. Heterogeneity in proliferation and cytoskeleton as well the capacity to remodel the extracellular matrix are maintained, when SMCs are suspended in PCLs.

Keywords: Lattice contraction; Extracellular matrix; Arterial wall; Proliferation; α-smooth muscle actin; α2 integrin; α1 integrin

Introduction

Accumulation of smooth muscle cells (SMCs) in the intima is a fundamental event in the pathogenesis of atherosclerosis and of restenosis after angioplasty [1,2]. The original hypothesis that all SMCs of the tunica media undergo phenotypic modulation and migrate into the intima [3,4] has been modified. Following the "monoclonal" hypothesis of Benditt and Benditt [5], successive studies report that SMC populations of human atheromatous plaque are oligoclonal [6,7]. This supports the hypothesis that a predisposed intrinsic or extrinsic cell subpopulation is responsible for the development of the intimal hyperplasia of atherosclerotic and restenotic processes [8–10]. Indeed, these SMC populations display heterogeneous biological properties [8,11]. It was reported that the media of the normal vessel wall contains the contractile as well as the synthetic SMC phenotypes [3,4]. Blood vessel-derived SMC populations can show distinct characteristics in vitro [12–14]. Heterogeneity is documented in rat SMCs from balloon injured carotid and aortic vessels. Spindle-shaped cells, showing "hill-and-

Abbreviations: Ab, antibody; α-SMA, α-smooth muscle actin; DMEM, Dulbecco's modified Eagle's medium; ECM, extracellular matrix; NB-SMC, newborn rat; FBS, fetal bovine serum; O-, 16–18 month old rats; PDGF, platelet-derived growth factor; PCL, populated collagen lattice; PDS, plasma-derived serum; SDS-PAGE, Sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SMC, smooth muscle cell; TEM, transmission electron microscopy; TI, thickened intima 15 days after ballooning; UM, underlying media; YA, young adult rats.

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valley" growth patterns are typical of cultured SMCs derived from normal media [15]. Cultured SMCs derived from the thickened intima 15 days after endothelial injury show an epithelioid SMC phenotype and a cobblestone morphology at confluence [9,13]. In addition, a small percentage of epithelioid clones can be obtained from rat aortic normal media [16]. SMCs derived from normal media require serum for growth, while SMCs derived from thickened intima (TI) can grow in the absence of serum [9,13]. SMCs cultured from normal media of arteries from young and old rats show differences in growth patterns [12,17]. Spindle-shaped SMCs growing in a "hill-and-valley" pattern predominate in cell cultures derived from newborn and young adult rat aorta. In contrast, epithelioid SMCs predominate in cell cultures derived from old rats, which also grow in "hill-and-valley" patterns and show greater proliferation than cells derived from young animals [12]. Dermal fibroblasts derived from the same rats show on opposite behavior, i.e., more proliferating in young compared to old animals, supporting the tissue specificity of this proliferative feature [12].

Arterial SMCs are surrounded and delimited in vivo by a complex extracellular matrix (ECM). Arterial SMCs synthesize ECM molecules that include different collagen types [18]. Interaction with collagen and other ECM components influences SMC proliferation. Integrins are a large family of cell adhesion molecules that include the receptors for cell–collagen interactions, which influence cell proliferation and differentiation [19,20]. The $\alpha 2\beta 1$ integrin is the major receptor for cell–collagen fibril interactions, whereas $\alpha 1\beta 1$ integrin mediates cell–monomeric collagen–matrix interactions [21]. SMCs in a free-floating populated collagen lattice (PCL) induce contraction and decrease in size over time [22,23]. We reported [13] that heterogeneous SMCs contract PCL differently. In this work, we have investigated some of the differences in proliferative capacity and cytoskeletal features of SMC populations when placed in collagen lattices.

Material and methods

Cell isolation and culture

SMCs were obtained by limited enzymatic digestion from the tunica media of thoracic aorta of 4-day-old newborn Wistar rats (NB-SMCs), 8- to 10-week-old rats, young adult (YA-SMCs) and 16- to 18-month-old rats (O-SMCs) as previously reported [12]. The endothelium of the thoracic aorta of 15 young adult Wistar rats was removed by ballooning [13]. Fifteen days after ballooning, SMCs were obtained from the thickened intima (TI)-SMCs and the underlying media (UM)-SMCs by enzymatic digestion, as previously reported [13]. Confluent SMC cultures were passed by trypsinization and maintained in Dulbecco's modified Eagle's medium (DMEM, Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS), and studied in their 3rd–5th passage.

Cell-populated collagen lattice manufacture and contraction

Collagen was purified from bovine tendon by limited pepsin digestion. Briefly, tendons were dissected from young calf hooves and cut into small pieces. All procedures were performed at 4°C. The tissue (1 g per 100 ml) was swollen overnight in 0.5 M acetic acid with stirring. The swollen tissue was homogenized, pepsin (Sigma) added at 10 mg per 100 ml and the mixture stirred for 2 days. The digest was centrifuged at 10,000 $\times$ g for 30 min, the insoluble pellet discarded, NaCl was added 10% w/v to the supernatant and the mixture stirred overnight. The insoluble collagen was collected by centrifugation and redissolved in 1.0 M NaCl, 50 mM Tris–HCl pH 7.5. The collagen solution was cleared of particulate matter by centrifugation and exhaustively dialyzed against 1 mM HCl. The clear viscous solution was frozen, lyophilized, weighted, dissolved in sterile 1 mM HCl at 5.0 mg/ml and stored at 4°C. The purity of the collagen was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), where all stained protein bands corresponded to collagen protein chains. After 24 h of serum starvation, SMCs freed from monolayer culture by trypsinization were counted. Aliquots of 200,000 cells in 3.0 ml of DMEM with 10% FBS (complete DMEM) were rapidly mixed with 1.0 ml of collagen solution (5 mg) and the mixture poured into a 60 mm Petri dishes. The dishes were placed in a humidified cell culture incubator at 37°C, where the collagen polymerized in less than 90 s, trapping cells within the newly polymerized matrix. The lattices were detached from the dish 1 h after casting. Other groups of SMC–PCLs were cast with DMEM supplemented with 2% plasma-derived serum (PDS) as previously reported [13]. To evaluate differences in SMC populations' capacity to contract free floating SMC–PCLs, the diameters of 2 ml SMC–PCLs cast in each well of 6-well plates were measured with a ruler to the nearest 0.5 mm over a period of 14 days. The area of each PCL was calculated and recorded. At day 5 and day 9, medium was removed and replaced with 2 ml of fresh complete DMEM or PDS supplemented DMEM. To verify the role of $\alpha 2$ and $\alpha 1$ integrins in the contraction, PCLs were cast with either complete or 2% PDS DMEM and 10 μg/ml hamster anti-$\alpha 2$ integrin antibody (Ab, clone Ha1/29, PharminGen, S. Diego, CA) or control hamster IgG (PharminGen). Contraction of PCLs cast in quadruplicate was measured after 24 h.

Smooth muscle cell proliferation, proliferation and cell cycle

Following changes in the numbers of SMCs incorporated in PCL in the presence of 10% FBS was measured by releasing cells by collagenase digestion. At days 2 and