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Enhanced neointimal fibroblast, myofibroblast content and altered extracellular matrix composition: Implications in the progression of human peripheral artery restenosis

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ABSTRACT

Background and aims: Neointimal cellular proliferation of fibroblasts and myofibroblasts is documented in coronary artery restenosis, however, their role in peripheral arterial disease (PAD) restenosis remains unclear. Our aim was to investigate the role of fibroblasts, myofibroblasts, and collagens in restenotic PAD.

Methods: Nineteen PAD restenotic plaques were compared with 13 *de novo* plaques. Stellate cells (H&E), fibroblasts (FSP-1), myofibroblasts (α-actin/vimentin/FSP-1), cellular proliferation (Ki-67), and apoptosis (caspase-3 with poly ADP-ribose polymerase) were evaluated by immunofluorescence. Collagens were evaluated by picro-sirius red stain with polarization microscopy. Smooth muscle myosin heavy chain (SMMHC), IL-6 and TGF-β cytokines were analyzed by immunohistochemistry.

Results: Restenotic plaques demonstrated increased stellate cells (2.7 ± 0.15 *vs.*1.3 ± 0.15) fibroblasts (2282.2 ± 85.9 *vs.* 906.4 ± 134.5) and myofibroblasts (18.5 ± 1.2 *vs.*10.6 ± 1.0) $p = 0.0001$ for all comparisons. In addition, fibroblast proliferation (18.4% ± 1.2 *vs.*10.4% ± 1.1; $p = 0.04$) and apoptosis (14.6% ± 1.3 *vs.*11.2% ± 0.6; $p = 0.03$) were increased in restenotic plaques. Finally, SMMHC (2.6 ± 0.12 *vs.*1.4 ± 0.15; $p = 0.0001$), type III collagen density (0.33 ± 0.06 *vs.* 0.17 ± 0.07; $p = 0.0001$), IL-6 (2.08 ± 1.7 *vs.*1.03 ± 2.0; $p = 0.01$), and TGF-β (1.80 ± 0.27 *vs.* 1.11 ± 0.18; $p = 0.05$) were increased in restenotic plaques.

Conclusions: Our study suggests proliferation and apoptosis of fibroblast and myofibroblast with associated increase in type III collagen may play a role in restenotic plaque progression. Understanding pathways involved in proliferation and apoptosis in neointimal cells, may contribute to future therapeutic interventions for the prevention of restenosis in PAD.

1. Introduction

Symptomatic peripheral arterial disease (PAD) restenosis remains a frequent complication of percutaneous intervention, increasing cardiovascular morbidity and mortality [1–5]. The specific mechanisms and histopathological findings leading to PAD restenosis involve inflammatory cell activation, cytokine production, smooth muscle cell (SMC) proliferation, apoptosis and collagen production [6]. Inflammatory cells like macrophages are well studied in coronary and peripheral arterial restenosis [7–9]. However, additional vascular cells may play a role in this proliferative process. Conventionally, resident intimal SMC proliferation plays a major role in restenosis [10]. SMCs include thin (α-actin positive) and thick (smooth muscle myosin heavy chain- SMMHC) filaments [11]. Other non-resident cells like fibroblast and myofibroblast, derived from the interstitial connective tissue beneath the subintimal layer of the atherosclerotic plaque, may contribute to the restenotic process. Fibroblasts and myofibroblasts may migrate

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into the neointima mediated by cytokines [12]. These cells along with the resident SMCs may proliferate and eventually undergo apoptosis mediated by caspase-3, and also contribute collagen deposition and neointimal expansion. Although caspase-3 plays a key role as effector in apoptosis, it also facilitates other cellular events in cell differentiation and development [13]. Furthermore, downstream effect of apoptosis is played by Poly (ADP-ribose) polymerase (PARP), DNA-binding enzyme which detects and signals DNA strand breaks [14]. PARP is activated at an intermediate stage of apoptosis and is then cleaved and inactivated at a late stage by caspase-3 and other proteases [14]. The biological presence of PARP indicates the cleaved end products of apoptosis mediated by caspase-3. In addition, interstitial fibroblasts and myofibroblasts synthesize the cytokines IL-6 and TGF-β [15]. IL-6 is involved in the migration of fibroblasts, and mediates signal transdifferentiation into myofibroblast [16,17]. In addition, TGF-β may contribute to transdifferentiation of the fibroblast into myofibroblast by inducing activation of α-smooth muscle actin (α-SMA) [18]. These epithelial mesenchymal transdifferentiation (EMT) from fibroblast to myofibroblast may contribute to extracellular matrix collagen synthesis [19–22]. Furthermore, resident neointimal SMCs and stellate cells will enhance neointimal collagen content. Therefore, neointimal fibroblast and myofibroblast may be crucial in type III collagen synthesis, leading to neointimal expansion [23,24]. These cells were extensively studied previously in coronary artery restenosis [25–29]. However, the role of fibroblast and myofibroblast in PAD restenosis has not been well studied. This study sought to investigate a possible association of increased fibroblast and myofibroblast composition with type III collagen deposition in restenotic plaques from patients with PAD.

2. Materials and methods

2.1. Tissue collection

A total of thirty-two plaque specimens were collected from twenty-four patients during atherectomy procedures using the SilverHawk atherectomy device (Fox Hollow Technologies, Redwood City, CA) at the cardiac catheterization laboratory, Mount Sinai Hospital. Nineteen restenotic plaque specimens were procured from 12 patients (8 female and 4 male) and thirteen control *de novo* plaques were procured from 12 patients (2 female and 10 male). All plaque samples were collected from superficial femoral arterial branch, and within 20 min, washed in saline and fixed in 10% buffered formalin and immediately submitted for processing into paraffin blocks. This study was approved by the institutional review board at the Icahn School of Medicine at Mount Sinai, New York. All the specimen procurements were done after informed consent was obtained for experimentation in human subjects. Also, this study conforms to the Declaration of Helsinki. Relevant demographic and clinical profiles were collected from case records and analyzed.

2.2. Quantification of stellate cell grade

Stellate cells were quantified using hematoxylin & eosin staining by evaluating cells resembling “stellate” shape appearance admixed in the loose myxoid and clear background (representing active myofibroblast) and histologically graded for the presence of stellate shaped cells per high-power field (HPF) as follows; Grade-1: scant to minimal stellate cells (occupying $\leq$ 25% of HPF), Grade-2: moderate stellate cells (occupying > 25%–75% of HPF), and Grade-3: dense stellate cells (occupying > 75% of HPF), in the neointima. A total of 10 randomly selected HPFs were used for the analysis.

2.3. Quantification of fibroblast content

Immunohistochemistry was performed using specific primary antibodies, rabbit polyclonal FSP-1 (ab27957, Abcam Inc., MA, 1:100 dilution) as a pan fibroblast marker. Appropriate secondary antibodies and positive (human skin) and negative (rabbit IgG from Dako, CA) controls were included to distinguish non-specific binding. Using FSP-1 immunostained sections, the total number of positively stained FSP-1 in ten random HPF (20X) for each plaque were enumerated. The total plaque area occupied in a HPF was measured in mm^2 using a computerized planimetry system. The density of FSP-1 positive stained cells was calculated by dividing the total number of FSP-1 positive stained cells by the total plaque area measured per HPF.

2.4. Quantification of smooth muscle cell myosin heavy chain (SMMHC) content

Immunochemistry was performed using specific, non-cross reacting rabbit polyclonal antibody against SMMHC (ab124679, Abcam, MA, 1:100 dilution). Appropriate positive controls for SMMHC (human colon) and negative controls (rabbit IgG from DAKO, CA) were included. Sections were examined under an Olympus BX 50 light microscope (Olympus America, Center Valley, PA), and the expression of SMMHC was quantified in 20 random high-power fields (HPFs) using percentage of positive cells stained per HPF. Using the intensity of cells immunostained with SMMHC, a semi quantitative score was used to grade as follows; grade 0: absent, grade 1: 25% stained, grade 2: 26%–50% stained, grade 3: >50 stained.

2.5. Quantification of myofibroblast content

Myofibroblasts are histologically defined as α-SMA positive cells, co-expressing fibroblast and vimentin markers. Myofibroblast cellular content was quantified using the percentage of cells expressing all these three markers. To detect myofibroblasts, immunofluorescent labeling with combined primary antibodies against the following antigens were adopted, rabbit polyclonal FSP-1 (ab27957-Abcam, MA, 1:100 dilution), mouse monoclonal α-SMA (α-SMA-FITC, F3777-Sigma Aldrich, MO, 1:500 dilution), and vimentin (ab45939-Abcam, MA, 1:100 dilution). Secondary antibodies, donkey anti-mouse Alexa Fluor 488 or anti-rabbit Alexa Fluor 594 (A-21202 and A-21207, respectively; Invitrogen, NY, 1:500 dilution) were used. Mounting medium containing DAPI (H-1200-Vector Lab, CA) was then applied. Quantification of the co-expression of α-SMA with FSP-1, and α-SMA with vimentin (myofibroblast) were performed in a blinded fashion. Cellular co-expression of randomly selected images captured at 20X magnification were quantified. Combined cellular co-expression of α-SMA with FSP-1 and α-SMA with vimentin (myofibroblast) were analyzed and scored as percentage of positive myofibroblast cell content. Images were acquired using the Leica TCS SP5 DMI, inverted confocal laser scanning microscope at Mount Sinai's Shared Resource Facility and analyzed using Leica LAS AF lite software system.

2.6. Quantification of cellular proliferation and apoptosis

Subgroup analysis from the same group of plaques in restenosis and *de novo* was performed for the intimal resident cells that proliferate and undergo apoptosis by quantifying the number of FSP-1 and α-SMA expressing cells that co-express Ki67 and caspase-3 by immunofluorescence staining and confocal microscopy. Primary antibodies against the following antigens were