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Suppressor of Cytokine Signaling-3 and Intimal Hyperplasia in Porcine Coronary Arteries following Coronary Intervention

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Abstract

Aims—The growth and differentiation of cells is regulated by cytokines by binding to cell-surface receptors and activating intracellular signal transduction cascade. Suppressor of cytokine signaling (SOCS)-3 is a negative regulator of cytokines. In this study we examined the expression of SOCS3 in porcine coronary artery smooth muscle cells (PCASMCs) *in vitro* and in proliferating smooth muscle cells of neointimal lesions after coronary artery intervention in a swine model.

Methods and Results—PCASMCs were cultured and stimulated with TNF- α and/or IGF-1 individually or in combination. Protein expression of SOCS-3 was examined using Western blot. For *in vivo* studies, six female Yucatan miniswine were fed with special high cholesterol diet for 8 months. At 4 months of high cholesterol diet, animals underwent coronary balloon angioplasty. At the end of 8 months animals were euthanized, coronary arteries were isolated and morphological and histological studies were performed. Western blot data revealed significantly high SOCS-3 expression in PCASMCs in the presence of either TNF- α or IGF-1 (5–6 fold) alone. However, in the presence of both TNF- α and IGF-1 the SOCS-3 expression was significantly decreased (4–5 fold). Results from morphological studies including, H&E and Masson's trichrome stain showed typical lesions with significant neointimal proliferation. Histological evaluation showed expression of smooth muscle α -actin and significantly increased proliferating cell nuclear antigen (PCNA) in neointimal lesion. Interestingly, there was significantly decreased expression of SOCS3 in smooth muscle cells of neointima as compared to control.

Conclusions—These data suggest that SOCS3 expression is decreased in proliferating smooth muscle cells of neointimal lesions. This leads to uncontrolled growth of vascular smooth muscle cells in injured arteries leading to restenosis. Therefore, local delivery of SOCS3 gene at the site of injury after coronary artery intervention could regulate the proliferation of vascular smooth muscle cells and help in preventing the neointimal hyperplasia and restenosis.

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Keywords

Angioplasty; Coronary Intervention; Inflammation; Intimal Hyperplasia; Restenosis; Suppressor of cytokine signaling-3

Introduction

It has been more than three decades since percutaneous transluminal coronary angioplasty (PTCA) was introduced for the treatment of coronary artery disease (CAD) (Hinohara, 2001). CAD outcome was improved in early years of invention of PTCA. However, formation of neointima following PTCA remains major limitation of this procedure. Accelerated atherogenesis following neointimal formation leads to thrombosis and re-occlusion which significantly contribute to high failure rate of this procedure (~50% within 1–10 years) (Jeremy and Thomas, 2010). Although the long-term outcomes have improved by the use of alternative techniques with stenting but, wider application of these devices is still limited due to restenosis (Goy and Eeckhout, 1998; Inoue et al., 2002; Roller et al., 2000; Roller et al., 2001). Even though incidence of restenosis has decreased with the use of drug-eluting stents, these drugs inhibit re-endothelialization of the stented segment making it more susceptible to thrombosis requiring longer period of anti-platelet therapy.

Inflammation plays a fundamental role in mediating all stages of atherosclerosis from initiation through progression and, ultimately, the thrombotic complications (Libby et al., 2002). TNF- α , a pluripotent inflammatory mediator (Jia et al., 2010) plays a significant role in the development of atherosclerosis through its mitogenic and pro-apoptotic effects by activating monocytes, macrophages and endothelial cells (Rakesh and Agrawal, 2005). Also, TNF- α controls the survival of SMCs through upregulation of FoxO family of transcription factors, which could potentially affect atherosclerotic plaque stability (Jia et al., 2010). However, the role of inflammation in neointimal thickening is not well established. Inflammatory reaction triggered by percutaneous intervention leading to intimal hyperplasia is even more prominent in atheromatous plaques which already has activated inflammatory cells (Toutouzas et al., 2004). TNF- α (Monraats et al., 2005) and the most potent smooth muscle cell growth factor, insulin-like growth factor-I (IGF-1) contribute to restenosis in atherosclerotic arteries following injury (Allen et al., 2005; Grant et al., 1999).

Tyrosine kinase family Jak activation and subsequent activation of STAT is a key event in signal transduction of different cytokines and growth factors (Heinrich et al., 2003; Ihle, 2001). These proteins regulate different biological processes including inflammation, cellular differentiation, and the proliferation (Ehling et al., 2007). Suppressor of cytokine signaling (SOCS) proteins, including SOCS-3, are eight structurally related proteins which decreases the Jak/STAT signaling by blocking the Jak tyrosine kinase activity and the activation of STAT factors (Ehling et al., 2007). However, there is limited information on the role of SOCS-3 in balloon angioplasty-induced neointimal proliferation. The burden of re-intervention due to restenosis is so high in CAD patients that there is strong need to study the pathogenesis of neointimal formation in further details. Due to unusual histological examination, studies for pathogenesis of restenosis are limited in humans. Animal studies permit fluctuation of experimental conditions and direct tissue examination. There is no perfect model to reiterate complex nature of coronary artery disease pathogenesis in animals. Many animal models including, rabbit, sheep and non-human primates have been used to examine fibroproliferative vascular disease. However, the swine model is comparable and similar in cardiovascular system to that of the human and in the injury response to interventional procedures and will mimic the real situation in patients with coronary artery

disease, and thus will make a direct contribution to understanding the pathophysiology of intimal hyperplasia and in-stent restenosis.

The purpose of this study was to examine the expression of SOCS3 in porcine coronary artery smooth muscle cells (PCASMCs) *in vitro* and in proliferating smooth muscle cells of neointimal thickenings after PTCA in a swine model.

Material and Methods

Isolation, Culture, and Treatment of Porcine Coronary Artery Smooth Muscle Cells (PCASMCs)

All experimental procedures were performed in accordance with the research protocol approved by the Institutional Animal Care and Use Committee of Creighton University. Pig hearts were obtained from a slaughter house and coronary arteries smooth muscle cells were cultured by the established protocol in our laboratory (Jia et al., 2007). Briefly, coronary arteries were dissected out from the heart and kept in smooth muscle cell media (SMCM; ScienCell, Carlsbad, CA) containing 1% penicillin/streptomycin solution (Sigma, St. Louis, MO) for 1 hour. Tissue was minced and washed with Dulbecco's modified eagle's medium (DMEM; Sigma, St. Louis, MO), incubated in 0.25% trypsin (1x) solution (Hyclone Lab, Logan, UT) for 30 minutes at 37°C and then washed with DMEM. Tissue was incubated in 0.2% collagenase for 3 hours at 37°C followed by washing with DMEM. Finally, tissue was rinsed with SMCM containing 10% fetal bovine serum (FBS) and seeded in 25 cm² cell culture flask (Corning Flask, Corning, NY) in 5 ml of SMCM with 10%FBS. Flasks were kept in an incubator at 37°C and 5% CO₂. Media was changed at every 48 hours and the cell growth was observed under inverted phase contrast microscope (Nikon, Japan). Once the flasks were confluent cells were split to next passage into 25 cm² cell culture flasks. The confluent cells showed the characteristic hill-and-valley pattern and spindle-shaped PCASMCs. The purity of the isolated VSMCs was confirmed with positive immunocytochemical staining of smooth muscle α -actin and myosin heavy chain.

The subcultured PCASMCs between passages 3–5 were used for in-vitro experiments. Prior to stimulation experiments, 70–80% confluent cells were serum starved for 24 hours with DMEM. The effect of TNF- α and IGF-1 was investigated individually or in combination on protein expression of SOCS-3 in PCASMCs. Cells were stimulated with TNF- α and IGF-1 for 24 hour at 100 ng/ml concentration. After 24 hours cells were harvested for protein isolation as per following protocol. Cells were rinsed with FBS free SMCM followed by incubation with 2 ml of 0.25% trypsin at 37°C for 5 minutes. SMCM (4 ml) containing 10%FBS was added to the flasks and cells were centrifuged at 1500 rpm for 5 minutes at 4°C. Media was removed and cell lysate was used for protein isolation. SOCS-3 expression was examined by Western blotting.

Protein Isolation, Quantification and Western Blot Analysis

Protein lysate was extracted using RIPA buffer (Sigma, St. Louis, MO) with protease inhibitor (Sigma, St. Louis, MO). Protein was quantified and each sample containing 30 μ g of protein was mixed with Laemmli buffer with 10% mercaptoethanol and proteins were resolved by electrophoresis using 10–20% polyacrylamide gels (BioRad, Hercules, CA). Proteins were transferred onto a nitrocellulose membrane (BioRad, Hercules, CA). The non-specific binding was blocked by incubating the nitrocellulose membrane in 5% non-fat dry milk for an hour. The membrane was then incubated overnight with rabbit polyclonal anti-SOCS3 antibody (1:1000) (ab 16030, Abcam) under gentle agitation at 4°C. Membrane was washed 6 times (10 minutes each) with washing buffer (0.05% tween-20 with PBS) to remove unbound primary antibody and then membrane was exposed to secondary antibody

linked to horseradish peroxidase, anti-rabbit, (1:2000; NB-730H, Novus Biologicals). Membrane was incubated for 1 hour at room temperature under gentle agitation. The membrane was washed three times with washing buffer and relative amount of protein staining was detected by ECL chemiluminescence detection reagents (Amersham Pharmacia Biotech, Piscataway, NJ). The emission was detected in EpiChemi darkroom and the image was captured with BioChemi CCD camera. Results were normalized against housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Animals

Institutional Animal Care and Use Committee of Creighton University approved all research protocols and the animals were housed in Animal Resource Facility of Creighton University, Omaha, NE and cared for as per National Institute of Health standards. Yucatan miniswine, weighing 30–40 lbs, were purchased from Lonestar Laboratories, (Sioux Center, IA).

Administration of high cholesterol diet

All animals were fed 1–1.5 lb/ animal/day special high cholesterol diet (Harlan Laboratories, Denver, CO) as reported earlier (Thorpe et al., 1996, 1998; Krueger et al., 2006). The major ingredients in the diet include: Corn 37.2% corn(8.5% protein), 23.5% soybean meal (44% protein), 20% chocolate mix, 5% alfalfa, 4% cholesterol, 4% peanut oil, 1.5% sodium cholate, and 1% lard. Blood (10 ml) was drawn every 15 day from the ear veins of animals for complete blood count (CBC) and complete metabolic and lipid profiles.

After 16 weeks animals were subjected to percutaneous transluminal balloon angioplasty (PTCA) in left anterior descending artery (LAD) or left circumflex artery (LCX).

Operative Procedure, Balloon Angioplasty, and Angiogram

Three days prior to the procedure, all animals were administered with aspirin (325mg/day) and ticlopidine (250mg/day). The day before the procedure, animals were fasted overnight and transferred to the surgical suite of the cardiovascular research lab. Swine were sedated by 0.1 ml/kg telazol/xylazine injected intramuscularly. Catheter was put in ear vein for intravascular fluid (Ringer Lactate). All animals were administered cefazolin (3–5mg/kg IM) to prevent infection. Animals were intubated and general anesthesia was induced with 4% Isoflurane. Temperature, heart rate, oxygen saturation, and capillary refill time was monitored during the surgical procedure. Femoral artery was exposed by cut down and 7F introducer was introduced. Heparin, 100U/kg, was administered i.v. before catheter introduction to maintain the blood flow. A 6F guide catheter was inserted and coronary vessels were visualized angiographically. Left anterior descending (LAD) or left circumflex (LCX) arteries were accessed for PTCA. After procedure was completed, femoral artery and the skin wound were sutured and the animal was transferred to the post-op room. To relieve pain, buprenorphine (0.1–0.3 mg/kg intramuscular) was given to all animals. Intravenous line was removed once animal gained sternal recumbency. Vitals were regularly monitored until animal was awake and walking. Next day, swine was transferred to animal facility. After coronary intervention all animals received aspirin (325 mg/day) and ticlopidine (250 mg/day) orally till they were euthanized.

At 8 and 16 weeks after the procedure, angiograms were obtained to check for intimal hyperplasia in coronary arteries. After performing the second angiogram animals were euthanized by administering high dose of barbiturates (Beuthanasia-D 1.0ml/10 lb i.v.).

Tissue harvest and processing

Heart was removed and placed in DMEM. Coronary vessels were dissected, removed and fixed in 4% formalin for 24 hours at room temperature. Tissues were embedded in paraffin

and thin sections (5µm) were obtained using microtome (Leica, Germany). Sections were stained with hematoxylin and eosin (H&E) and trichrome stain for histomorphometric studies.

Immunohistochemistry

Paraffin embedded samples, after deparaffinization and rehydration, were treated by steam heating for antigen retrieval (20–30 min) using DAKO antigen retrieval solution (DAKO, Carpinteria, CA). Slides were washed using phosphate buffered saline (PBS) twice 5 min each. Endogenous peroxidase was inhibited by immersing the slides in a 3% hydrogen peroxide solution for 20 min. Slides were then washed twice for 5 min in PBS. After pre-incubation of the tissue sections with 10% serum for 1 h to avoid nonspecific binding (VECTASTAIN Elite ABC system, Vector Laboratories, Burlingame, CA), the sections were incubated with primary antibodies against proliferating cell nuclear antigen (PCNA) (mouse anti-PCNA; sc-25280; Santa Cruz Biotech) overnight at 4°C. Antibodies were diluted (1:200) with PBS. Slides were washed twice with PBS and consecutively incubated with biotinylated secondary antibody for 1 hour. Slides were washed twice with PBS and incubated with ABC solution (VECTASTAIN Elite ABC system, Vector Laboratories, Burlingame, CA) for 30 min. Slides were washed twice again with PBS and finally incubated with diaminobenzidine (Vector Laboratories, Burlingame, CA) for 1 min. Immediately after staining, slides were washed with distilled water for 5 min and counterstained with hematoxylin for 7 seconds. Slides were rinsed for 5 min with distilled water and dehydrated for 3–5 min each with 70–100% isopropanol. Finally, samples were immersed in xylene for 5 min each and mounted by using permount (Fisher Scientific, Pittsburg, PA). Sections incubated without the primary antibody served as negative controls. Slides were examined using an inverted microscope (Olympus) and saved as jpg-files.

Immunofluorescence

After deparaffinization and rehydration, antigen retrieval was performed prior to immunostaining. Sections were incubated for 2 hr in block/permeabilizing solutions containing PBS, 0.25 % Triton X-100, 0.1 bovine serum albumin (BSA), and 5% (v/v) goat serum at room temperature. The slides were subsequently incubated with primary antibody solutions including mouse anti-smooth muscle alpha actin (α -SMA) (Santacruz biotech, Sc-58669), rabbit anti-SOCS-3 (Abcam; Ab16030) (1:100 antibodies, PBS, 0.1% Triton X-100, 10 mg/ml BSA and 1% goat serum) at 4°C overnight. After washing with PBS containing 0.1 % BSA three times for 5 minutes each, a secondary antibody (affinity purified goat anti-mouse and goat anti-rabbit cyanine 3 (cy3) antibody, 1:200) (Jackson ImmunoResearch, Westgrove, PA) was applied to the sections for 1 hr in dark. Negative controls were run in parallel with normal host IgG including chromPure mouse IgG, and ChromPure rabbit IgG or complete omission of primary antibody. Sections were washed with PBS three times for 5 min. Nuclei were counterstained with 4', 6- diamidino-2-phenylindole (DAPI). A single layer of nail polish was placed around the edge of slide to prevent escape of mounting media from the coverslip. Pictures were taken within 1 hr of mounting using Olympus DP71 camera.

Statistical analysis

Data was analyzed using GraphPad Prism 5.0 biochemical statistical package (GraphPad Software, Inc., San Diego, CA). Values of all measurements were expressed as mean $\pm$ SEM. Statistical analysis was performed using one-way ANOVA with Bonferroni's multiple comparison test to analyze statistically significant differences between groups. Differences at $p < 0.05$ were considered significant. The semi-quantitative scoring of immunostaining was performed as follows: 0- undetectable, 1+- weakly positive, 2+- moderately positive, 3+- strongly positive.

Results

Effect of TNF- α , IGF-1 and TNF- α + IGF-1 treatment on SOCS-3 protein expression in PCASMCs

The stimulation of the PCASM cells with TNF- α or IGF-1 (100ng/ml) for 24 h significantly increased (~5–6 fold) SOCS-3 protein expression (Fig. 1). However, stimulation of the cells with both TNF- α and IGF-1 (each 100ng/ml) significantly downregulated SOCS3 expression (~5 fold) (Fig. 1)

Vessel damage and neointimal development after balloon angioplasty

Balloon injury resulted in medial rupture in all balloon-injured coronary arteries. Significant neointimal proliferation was observed both in left anterior descending and left circumflex arteries following PTCA. Morphometric analysis of coronary artery tissue sections was performed using Scion image software for windows by Scanalytics. The area within the lumen (LA) and within internal elastic lamina (IEL) was determined. Percent restenosed area was calculated by dividing the area within IEL with LA. Comparing the luminal area, significant restenosis was observed in coronary arteries with balloon angioplasty compared to uninjured vessel (50.4 ± 4.1) (Fig. 2A, B). Masson's trichrome staining showed profuse collagen in neointima as well as adjacent adventitia (Fig. 2C).

Vascular injury, PCNA, and α -SMA

Immunohistological analysis of proliferating cell nuclear antigen (PCNA) was done to examine proliferating cells in neointimal area. Smooth muscle cells (SMCs) of neointimal lesion showed increased PCNA expression than normal SMCs in the media. Expression of smooth muscle α -actin (α -SMA) was also examined in neointimal lesions by immunofluorescence. Strong expression of α -SMA in these lesions confirmed that vascular SMCs were the main cellular component of neointimal proliferative lesions (Fig. 3 C–F). These findings were consistent across multiple samples from four different animals.

Vascular injury and SOCS-3 expression

Balloon-injured arteries were examined for SOCS3 expression by immunofluorescence. There was significantly decreased expression of SOCS3 in smooth muscle cells of neointimal region than in the normal media in all tissue sections from four individual animals (Fig. 4). These results suggest a strong association between vascular injury and SOCS-3 expression in coronary arteries following balloon angioplasty.

Discussion

Role of inflammation in the development of atherosclerosis has been studied widely and it has been shown that inflammation plays a key role in the pathogenesis of atherosclerosis (Hansson, 2005; Libby, 2002). However the relationship between inflammation and restenosis is still controversial. There is a significant increase in the markers of inflammation after percutaneous coronary intervention (Lincoff et al., 2001). In this study, we found that the expression of SOCS-3 was significantly reduced in balloon-injured porcine coronary arteries. We have also found that the stimulation of PCASMCs with TNF- α and IGF-1 under *in-vitro* conditions significantly increased SOCS-3 expression. Interestingly, stimulation of PCASMCs with both TNF- α and IGF-1 at the same time significantly abolished SOCS-3 expression.

TNF- α is a strong pro-inflammatory mediator cytokine (Bedoui et al., 2005). A number of studies have shown that TNF- α induces SOCS-3 expression in human or rat liver macrophages (Bode et al., 1999; Dalpke et al., 2001; Stoiber et al., 1999). The results from

our studies that stimulation of the cells with TNF- α result in SOCS-3 upregulation in PCASMCs are in accordance with these studies. Another study has reported that TNF- α increases SOCS-3 expression in human macrophages by stabilizing mRNA of SOCS-3 (Ehltting et al., 2007). Although now there is clear evidence that level of TNF- α is augmented after percutaneous coronary intervention (Kozinski et al., 2005) but it does not act alone to induce neointimal formation (Miller et al., 2005). This could be supported by the findings that infusion of TNF- α (2ng/min) to the injured artery immediately after angioplasty procedure significantly increased expression of both intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 but it did not alter the extent of neointimal formation observed 8 days after injury (Miller et al., 2005).

Insulin-like growth factor-1 (IGF-1) is a potent endocrine and autocrine/paracrine mitogenic factor that exerts pleiotropic effects on cells involved in atherogenesis (Shai et al., 2010). In a recent study, mechanical stretch stimulus causes proliferation of venous smooth muscle cells through IGF-1 receptor activation (Cheng and Du, 2007). Also, increased proliferation and IGF-1 expression in rat aortic SMCs after cyclic stretch and anti-IGF-1 antibody prevented stretch induced proliferation (Standley et al., 1999). Studies in experimental *in vivo* models have also demonstrated the role of IGF-1 in cardiovascular diseases (Frystyk et al., 2002). These findings support the role of IGF-1 in cell proliferation following injury. However, interaction of SOCS-3 and IGF-1 has not yet been studied. SOCS3 is usually upregulated to suppress the effect of inflammatory cytokines (Rakesh and Agrawal, 2005). Results from our studies show that SOCS-3 expression is significantly enhanced in PCASMCs not only after stimulation with TNF- α but also following IGF-1 stimulation. Increased expression of inflammatory cytokines involved in acute and chronic complications of coronary angioplasty causes release of vasoactive substances and induces growth factors (Hojo et al., 2000). Since both cytokines and growth factor are released during coronary intervention, we analyzed the effect of TNF- α and IGF-1 on the expression of SOCS-3 in PCASMCs. Interestingly, treatment of the cells with both TNF- α and IGF-1 at the same time significantly reversed the enhanced SOCS-3 expression due to TNF- α or IGF-1 alone. Since both mitogens and inflammatory cytokines could be released simultaneously under *in vivo* conditions during coronary interventional procedures, our novel findings under *in vitro* conditions led us to hypothesize that SOCS3 expression in coronary arteries decreases following balloon angioplasty or any other interventional procedure.

The coronary vascular injury models in swine, by either overstretching injury alone or stenting, are well established to study potential restenosis therapies (Suzuki et al., 2008). Histomorphometric analysis from our study demonstrated significant neointimal formation in coronary arteries. Immunohistochemical analysis showed significantly decreased expression of SOCS-3 in proliferating smooth muscle cells of neointimal lesions. These novel findings supported the results from our *in vitro* studies. Thus, decreased expression of SOCS-3 in post angioplasty coronary arteries suggest that TNF- α and IGF-1-induced silencing of SOCS-3 could lead to uncontrolled growth of vascular smooth muscle cells in injured arteries leading to intimal hyperplasia and restenosis. Further studies are warranted to elucidate the underlying mechanism. Due to accessibility of the site of treatment, restenosis after balloon angioplasty has been a striking target for gene therapists. Therefore, local delivery of SOCS-3 gene at the time of coronary intervention could be a potential therapeutic strategy to regulate the proliferation of vascular smooth muscle cells and help in preventing the neointimal formation and restenosis.

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Figure 1.

Effect of TNF- α and IGF-1 stimulation on SOCS-3 protein expression in PCASMCs. PCASMCs were serum starved for 24 hours followed by stimulation with TNF- α (100ng/ml) and/or IGF-1(100ng/ml). Protein was isolated from cell lysates and subjected to immunoblotting using SOCS-3 antibody. The relative amount of SOCS-3 protein expression was measured by including GAPDH as a loading control. The Western blot data is a representative of three individual experiments. Data shown are mean $\pm$ SEM (N = 3). *p<0.05, **p< 0.01, ***p< 0.001 compared to control group.

Figure 2.

Photomicrographs of swine coronary artery tissue sections. Paraffin embedded thin sections were cut, deparaffinized and stained with H&E and Masson's trichrome stain. A: H&E staining of normal uninjured coronary artery, B and C: Restenotic coronary artery, 16 weeks after balloon angioplasty. Balloon angioplasty was performed in coronary artery as discussed in the Methods section. In the H&E staining, the fracture in the internal elastic lamina (IEL) and neointimal thickening were observed (B). In the Masson's trichrome staining of the tissue sections, collagen deposition in neointimal area was prominently present (C). Magnification (100× – 200×); L: lumen, M: medial layer, N: neointima

Figure 3.

Photomicrographs showing expression of proliferating cell nuclear antigen (PCNA) and smooth muscle alpha-actin (α -SMA) in thin sections of post balloon angioplasty swine coronary arteries. A–B: Immunohistochemical expression of PCNA at low (100 $\times$) and high (400 $\times$) magnification. Sections were stained with DAB as chromogen and counterstained using hematoxylin. Increased PCNA expression in the neointimal area was found. Arrows indicate proliferating cells. C–D: Immunofluorescence of α -SMA expression in post balloon angioplasty coronary artery at low (100 $\times$) and high (200 $\times$) magnification. Sections were stained using mouse anti- α -SMA antibody and goat anti-mouse cy3 as secondary antibody. DAPI was used to stain the nuclei. E–F: DAPI overlay with mouse anti- α -SMA antibody

and goat anti-mouse cy3 as secondary antibody at 100× and 200× magnification. Strong expression of α -SMA was found in the neointimal area. L: lumen, M: medial layer, N: neointima

Figure 4.

Photomicrographs showing expression of suppressor of cytokine signaling (SOCS)-3 in the swine coronary arteries post balloon angioplasty. Paraffin embedded thin sections were cut, deparaffinized and stained with rabbit anti-SOCS-3 primary antibody and goat anti-rabbit cy3 secondary antibody. Nuclei were counterstained with DAPI. A and C: (100×) cy3 SOCS-3 expression. B and D: (200×) DAPI overlay. There was significantly reduced expression of SOCS-3 in restenotic area. L: lumen, M: medial layer, N: neointima