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Expression and Suppressive Effects of Interleukin-19 on Vascular Smooth Muscle Cell Pathophysiology and Development of Intimal Hyperplasia

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Anti-inflammatory cytokines may play a protective role in the progression of vascular disease. The purpose of this study was to characterize interleukin (IL)-19 expression and function in the development of intimal hyperplasia, and discern a potential mechanism of its direct effects on vascular smooth muscle cells (VSMCs). IL-19 is an immunomodulatory cytokine, the expression of which is reported to be restricted to inflammatory cells. In the present study, we found that IL-19 is not expressed in quiescent VSMCs or normal arteries but is induced in human arteries by injury and in cultured human VSMCs by inflammatory cytokines. Recombinant IL-19 significantly reduced VSMC proliferation ($37.1 \pm 4.8 \times 10^3$ versus $72.2 \pm 6.1 \times 10^3$ cells/cm²) in a dose-dependent manner. IL-19 adenoviral gene transfer significantly reduced proliferation and neointimal formation in balloon angioplasty-injured rat carotid arteries (0.172 ± 29.9 , versus 0.333 ± 71.9 , and $0.309 \pm 56.6 \mu\text{m}^2$). IL-19 induced activation of STAT3 as well as the expression of the suppressor of cytokine signaling 5 (SOCS5) in VSMCs. IL-19 treatment significantly reduced the activation of p44/42 and p38 MAPKs in stimulated VSMCs. Additionally, SOCS5 was found to interact with both p44/42 and p38 MAPKs in IL-19-treated human VSMCs. This is the first description of the expression of both IL-19 and SOCS5 in VSMCs and of the functional interaction between SOCS5 and MAPKs. We propose that through induction of SOCS5 and inhibition of signal transduction, IL-19 expression in VSMCs may represent a novel, protective, autocrine response of VSMCs to inflammatory stimuli. (*Am J Pathol* 2008, 173:901–909; DOI: 10.2353/ajpath.2008.080163)

Despite recent advances, the efficacy of vascular interventional procedures such as balloon angioplasty is lim-

ited because of the high occurrence of vascular intimal hyperplasia observed in a significant number of patients undergoing these procedures.^{1,2} The high incidence of transplant vasculopathy is also the major complication that limits long-term survival of solid organ transplantation.² Common to both of these vasculopathies is a localized inflammatory reaction that elicits activation of normally quiescent medial vascular smooth muscle cells (VSMCs).³ As part of the response to injury, activated VSMCs migrate from the media into the lumen of the vessel where they proliferate and synthesize cytokines that they respond to in an auto-crine manner, sustaining the progression of intimal hyperplasia.³ Restenosis, atherosclerosis, and many other vascular diseases are inflammatory in nature, and ultimately effect VSMC as the effector cell.

The deleterious effects of pro-inflammatory and proliferative cytokines such as tumor necrosis factor- α , interleukin (IL)-1 β , and platelet-derived growth factor (PDGF) on VSMC pathophysiology and development of intimal hyperplasia has been well documented.^{3–5} Although a great deal of attention has been given to the negative effects of pro-inflammatory cytokines, little has been reported on the potential protective effects of anti-inflammatory cytokines on the vascular response to injury. Most of the emphasis on secretion of inflammatory mediators has been placed on leukocytes, and the role of nonimmune cells in this process is poorly understood. In particular, the direct cellular and molecular effects of anti-inflammatory cytokines on VSMC pathophysiological processes remains relatively uncharacterized.

Interleukin-19 (IL-19) is a recently described IL-10 family member that is basally detected in human monocytes, B lymphocytes, and T lymphocytes.⁶ IL-19 can be up-regu-

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lated in monocytes, B lymphocytes, and T lymphocytes by lipopolysaccharide treatment and G-CSF.⁷ IL-19 expression is reported to be restricted to immune cells, and all of our knowledge concerning the function of this cytokine comes from experiments performed in inflammatory cells. IL-19 has been ascribed to be an anti-inflammatory cytokine because IL-19 treatment of maturing antigen-presenting cells promote the Th2 (regulatory) T cell response, rather than the Th1 (T helper) response.⁸ This Th2/Th1 shift biases toward a more anti-inflammatory phenotype. IL-19 treatment increases IL-4 and decreases interferon- γ in regulatory T cells, and induces secretion of IL-10 in human peripheral blood mononuclear cells.^{8,9}

We detected IL-19 mRNA expression in primary human VSMCs in response to inflammatory stimuli using cDNA microarray analysis.¹⁰ This was unexpected because nothing has been published concerning the expression or presumed function of IL-19 in VSMC pathophysiology, or a role in modulation of vascular diseases. From its proposed anti-inflammatory activity in modulation of immune cells, we hypothesized that cytokine-inducible expression of IL-19 by VSMCs could be a beneficial autoregulatory feedback mechanism whereby the injured artery could dampen inflammation. The overall goal of the present study is to characterize the expression of IL-19, and its potentially suppressive effects on activation of VSMCs and development of intimal hyperplasia. Using *in vivo* gene delivery, we report that IL-19 attenuates balloon angioplasty-induced intimal hyperplasia. Using *ex vivo* mechanistic approaches, we report that IL-19 is expressed in stimulated VSMCs and suppresses proliferation and MAPK signal transduction by *de novo* synthesis of the inhibitory protein SOCS5.

Materials and Methods

Cells and Culture

Human coronary VSMCs were obtained as cryopreserved secondary culture from Cascade Corporation (Portland, OR) and cells from passages three to six were used as described previously.¹⁰ Platelet-derived growth factor AB (40 ng/ml), interferon- γ (100 U/ml), and tumor necrosis factor- α (1.0 ng/ml) were purchased from Sigma (St. Louis, MO). T-lymphocyte-conditioned medium was used at 5% and purchased from Fisher Biotech (Pittsburgh, PA). Recombinant IL-19 (12 nmol/L to 1.2 μ mol/L) was purchased from Fitzgerald Industries, Concord, MA. For proliferation, equal numbers of VSMCs were seeded into 24-well plates at a density of 3000 cells per cm², in growth media, in the presence and absence of IL-19, and were counted in the presence of trypan blue on the 2nd, 4th, 5th, and 7th day using a standard hemocytometer as described.¹¹ For gene expression analysis screen, VSMCs were incubated in serum-reduced media [0.25% fetal calf serum (FCS)] for 48 hours, at which time one sample was stimulated with 120 nmol/L IL-19. RNA isolation and nylon cDNA microarrays specific for JAK/STAT gene expression (no. OHS-039) were purchased from SuperArray, Inc. (Frederick, MD) and performed as described.¹¹ Changes in gene expression were

detected by autoradiography, and quantitated by scanning densitometry from film exposed in the linear range of detection. Expression was normalized to three different housekeeping genes included on the membrane.

Western Blotting and Co-Immunoprecipitation

Human VSMC extracts were prepared as described.¹¹ Membranes were incubated with a 1:2000 dilution of primary antibody, and a 1:3000 dilution of secondary antibody. Equal protein concentrations of cell extracts were determined by the Bradford assay and equal loading on gels was verified by Ponceau S staining of the membrane, and reactive proteins visualized using enhanced chemiluminescence (Amersham, Arlington Heights, IL) according to the manufacturer's instructions. Signal was normalized with GAPDH, or in the case of phosphorylation, total antibody. Anti-total and phospho STAT3, p44/42, and p38 MAPK (primary rabbit) were purchased from Cell Signaling, Beverly, MA; anti-SOCS5 and GAPDH from Santa Cruz Biotechnology (Santa Cruz, CA); and anti-IL19 from R&D Systems (Indianapolis, IN). For STAT3 translocation, VSMCs were serum-starved for 24 hours and then stimulated with IL-19 for the times indicated. Lysates were made using the Compartmental Protein Extraction kit from Chemicon International (Temecula, CA) according to the manufacturer's instructions. For co-immunoprecipitation, VSMCs were serum-starved, pretreated with IL-19 for 8 hours to induce endogenous SOCS5 expression, and then 15 minutes with PDGF so that MAPK would be phosphorylated. Both forward and reverse immunoprecipitation was performed on the same gel. Lysates were precleared with protein A/G Sepharose and then incubated overnight with either mouse SOCS5, p44/42, p38 antibody, or irrelevant, but identical isotype antibody (control) and immunoprecipitated. Immunoprecipitates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to nitrocellulose membrane. The membrane was cut into sections based on molecular weight of the protein being detected, and immunoblotted with either rabbit SOCS5, p44/42, or p38 antibody. Positive antibody controls for Westerns were 10% of the lysate volume run as a lane on the immunoprecipitation gel.

Adenovirus

The human IL-19 cDNA clone was obtained from Origene, Inc. (Rockville MD). The open reading frame was inserted into the pShuttle vector and high-titer stocks prepared by Vector Labs, Inc. (Philadelphia, PA). AdLacZ, in the same viral backbone, was purchased from Vector Labs.

Rat Left Common Carotid Artery Balloon Angioplasty and Gene Transfer

Left common carotid artery balloon angioplasty was performed on 350 g, male, Sprague Dawley rats (Charles River Breeding Laboratory Inc., Wilmington, MA) under sodium pentobarbital anesthesia (65 mg/kg, i.p.) as de-

scribed previously.¹² Briefly, the left external carotid artery was cleared of adherent tissue allowing insertion of an 2-F Fogarty arterial embolectomy catheter. The catheter was guided down the common carotid artery to the aortic arch, inflated with a fixed volume of fluid, and withdrawn back to the site of insertion a total of three times. After balloon injury, a cannula was introduced into the common carotid artery and the distal injured arterial segment isolated by temporary clips placed midway in the injured segment and at the orifice of the internal carotid artery. This space was filled with the appropriate adenovirus (final titer = 1.0×10^8 pfu/ml). Incubation was allowed to proceed for 15 minutes and then the solution was retrieved, the cannula removed, blood circulation restored, and the wound closed as described.¹² The vessels were harvested 14 days later for morphology, 7 days for quantitation of proliferation, inner perfusion fixed in buffered 10% formaldehyde solution (pH 7.4), paraffin-embedded, sectioned (5 μ m), and processed for histology. All surgical procedures were performed in accordance with the guidelines of the Animal Care and Use Committee of Temple University and the American Association for Laboratory Animal Care.

Immunohistochemistry and Morphometry

Immunohistochemistry was performed as described.¹¹ Coronary arteries from transplanted hearts with severe transplant vasculopathy (TA) were excised from patients ($n = 3$) at the time they received their second transplanted heart. Normal coronary arteries ($n = 3$) were taken from nonfailing hearts of individuals that died of accidents and wounds, but the heart not used for transplant. Five- μ m sections from injured rat arterial tissue fixed in methanol were deparaffinized and blocked in 10% goat serum. Sections were incubated with primary rabbit antibody; anti-IL-19, Ki-67, leukocyte common antigen (LCA), or mouse SMC actin, at 1 μ g/ml in 1% bovine serum albumin/phosphate-buffered saline (PBS), and were applied for 1 hour, followed by biotinylated secondary antibody (1:200), followed by avidin-biotin-peroxidase complex each for 30 minutes. Nonspecific isotype antibodies were used as negative controls. Staining was visualized with the substrate diaminobenzidine (Vector) producing a reddish-brown color and then counterstained with hematoxylin. LCA and SMC actin antibodies were from Lab Vision, Inc. (Freemont, CA) For quantitation of intimal hyperplasia, digitized images were measured and averaged from at least three representative 5- μ m-thick stained tissue sections at least 100 to 150 μ m apart per carotid artery using Image Pro Plus (Media Cybernetics, Bethesda, MD). Six animals per condition were used. The circumference of the lumen, the area encircled internal elastic lamina, and the external elastic lamina were quantitated. The medial area was calculated by subtracting the area defined by the internal elastic lamina from the area defined by the external elastic lamina, and intimal area calculated as the difference between the area inside the internal elastic lamina and the luminal area using an automated computer-based image

analyzer (Image-Pro Plus, Diagnostic Instruments, Inc, Bethesda, MD). For quantitation of *in vivo* proliferation, sections from 7-day injured rats were co-stained with the nuclear proliferation marker anti-Ki-67, and anti-SMC- α actin, secondary antibody was conjugated to Alexa Fluor 568 (red) and Alexa Fluor 488 (green) (Molecular Probes, Inc., Eugene, OR). Ki-67-positive, SMC actin-positive cells in the media and neointima were counted from four merged images per section, from at least four representative 5- μ m-thick stained tissue sections at least 100 to 150 μ m apart from each other per carotid artery.

Statistical Analysis

Results are expressed as mean $\pm$ SE. Differences between groups were evaluated with the use of analysis of variance, with the Newman-Keuls method applied to evaluate differences between individual mean values and by paired *t*-tests where appropriate, respectively. Differences were considered significant at a level of $P < 0.05$.

Results

Expression of IL-19 in Injured Arteries and Stimulated, but Not Quiescent VSMCs

We detected IL-19 mRNA expression in primary human VSMCs in response to inflammatory stimuli using cDNA microarray analysis.¹⁰ Western blot confirmed this inducible expression (Figure 1A) showing that IL-19 is not normally expressed in VSMCs, but can be induced by 10% FCS. This is unexpected in that IL-19 expression had previously been ascribed to be restricted to immune cells. IL-10 mRNA and protein are not detectable in quiescent or stimulated VSMCs (not shown). Immunohistochemical analysis demonstrated that IL-19 can be detected in medial and neointimal VSMCs in a human coronary artery with transplant vasculopathy, but not an uninjured, normal human artery (Figure 1B). Further, immunohistochemistry verifies that IL-19 expression co-localizes with SMC- α -positive VSMCs (Figure 1C). Not unexpectedly, IL-19-positive cells also co-stain with leukocyte common antigen-positive cells in these arteries. This is the first demonstration of IL-19 expression in VSMCs, and suggests that IL-19 is an inflammation-responsive cytokine in activated VSMCs.

It was important to characterize the kinetics, and localize accumulation of IL-19 protein in injured arteries. We used the well-characterized rat carotid artery balloon angioplasty model and immunohistochemistry for this experiment.^{13,14} No IL-19 expression is detectable in uninjured, sham-operated arteries, but IL-19 is rapidly detectable in medial VSMCs as early as day 1 after angioplasty injury (Figure 2). Although expression remains detectable in 7- and 14-day injured arteries, higher expression immunolocalized to the developing neointima. This indicates that IL-19 expression is rapidly inducible by mechanical injury of VSMCs, as well as by immunological injury indicative of transplant vasculopathy.

Figure 1. Expression of IL-19 in VSMCs. **A:** Representative immunoblot of human coronary artery VSMCs that were serum-starved for 48 hours and then stimulated with the factors shown for 48 hours. Extracts were immunoblotted with anti-IL-19 or GAPDH antibody. TCM, T-cell conditioned media; IFN- γ , interferon- γ ; PDGF, platelet-derived growth factor; TNF- α , tumor necrosis factor- α . **B:** Immunohistochemical analysis of IL-19 expression in injured arteries. Sections from vessels obtained from a normal heart without coronary artery disease, and a rejected heart from a patient with transplant vasculopathy stained with IL-19 antibody. Red-brown staining indicates antibody recognition. Sections were counterstained with hematoxylin. **C:** IL-19 co-localizes with VSMCs and leukocytes. Immunofluorescence immunohistochemistry of an artery from a human patient with transplant vasculopathy co-stained with anti-IL-19 (red), SMC- α actin, or CD45 leukocyte common antigen (both green). Scale bar = 50 μ m. Original magnifications: $\times 400$ (**B**); $\times 600$ (**C**).

Antiproliferative Effects of IL-19 on VSMCs

Nothing has been reported on the effects of IL-19 on VSMC pathophysiological processes. One study has reported anti-proliferative effects of IL-10 on VSMCs,¹⁵ and we hypothesized the same would be true for IL-19. Figure 3A demonstrates a dose-dependent, anti-proliferative effect of IL-19 on primary human coronary artery VSMCs (72.2 ± 6.1 , versus 55.34 ± 3.9 , 44.3 ± 4.9 , $37.1 \pm 4.8 \times 10^3$ cells/cm² for control, and 12 nmol/L, 120 nmol/L, and 1200 nmol/L IL-19, respectively; $P < 0.05$ to 0.0001) at 6 days. Based on the growth curve, recombinant IL-19 growth-inhibitory effects appear to be greatest early after IL-19 addition. We constructed IL-19 expressing adenovirus (AdIL-19), and observed that human VSMCs infected with this adenovirus also show reduced proliferation (35.0 ± 5.4 versus 53.0 ± 3.8 and $54.57 \pm 3.9 \times 10^3$

Figure 2. Kinetics of IL-19 expression in injured arteries. Rat carotid arteries were subject to balloon angioplasty, and harvested at different times after the procedure. IL-19 expression was detected by immunohistochemistry. Red-brown staining indicates positive staining, and sections were counterstained with hematoxylin. Medial VSMC, m; neointimal, ni. Scale bar = 50 μ m.

cells/cm² for AdIL-19, AdLacZ, and untreated control VSMCs, respectively) at 6 days (Figure 3B). These cells express IL-19 (Figure 3C). This reduction is not a result of apoptosis or cell death, as indicated by trypan blue exclusion. VSMCs treated with recombinant IL-19 and AdIL-19 were also negative for active caspase 3, indicating that the reduction in cell numbers was not because of induction of apoptosis (data not shown). These data indicate that IL-19 has anti-proliferative effects for cultured VSMCs.

Effects of IL-19 Expression on Proliferation and Development of Neointimal Hyperplasia

It was important to determine a direct relationship between IL-19 expression and development of proliferative intimal hyperplasia. Considering its direct immunomodulatory effects on inflammatory cells, it was important, as much as possible, to limit expression of IL-19 to VSMCs. Balloon angioplasty was performed on rat carotid arteries and 1×10^8 PFU of AdIL-19, AdLacZ, or vehicle only delivered and the effects of gene delivery on intimal hyperplasia determined after 14 days. Successful gene transfer was detected by immunohistochemistry and immunoblotting, and demonstrated overexpression of IL-19 in the intima and medial VSMCs of adenoviral-infected carotid arteries (Figure 4). Endogenous IL-19 was expressed almost exclusively in neointimal cells in control arteries. Delivery of AdIL-19 significantly decreased development of neointimal hyperplasia ($n = 6$, $P < 0.001$). Neointimal area was 0.172 ± 29.9 , 0.333 ± 71.9 , and $0.309 \pm 56.6 \mu\text{m}^2$ for AdIL-19, AdLacZ, or PBS vehicle-

Figure 3. IL-19 is anti-proliferative for VSMCs. **A:** Equal numbers of human VSMCs were seeded into 24-well trays and stimulated with the indicated amounts of IL-19 for the indicated times. **B:** VSMCs were infected with 50 MOI of AdLacZ or IL-19. The next day, VSMCs were seeded into 24-well trays, and counted at the designated times. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.0001$ versus control cells for all experiments ($n = 3$). **C:** Relative expression of IL-19 in adenoviral-infected VSMCs. Extracts from adenoviral-infected VSMCs were separated by SDS-PAGE, and IL-19 or GAPDH protein was detected by Western blot.

treated carotid arteries, respectively. The neointimal:medial ratio for these animals was 0.37 ± 0.05 , 0.94 ± 0.08 , and 0.89 ± 0.7 , for AdIL-19, AdLacZ, or PBS vehicle-treated carotid arteries, respectively. The lumen area was significantly larger for the AdIL-19-infected arteries compared with controls; 1096 ± 148.1 , 505.7 ± 119.6 , and $610.9 \pm 98 \mu\text{m}^2$ for AdIL-19, AdLacZ, or PBS, respectively. No differences in medial area or circumference between experimental groups was noted.

To determine effects on VSMC proliferation, sections from animals injured 7 days previously were co-stained with anti-Ki-67, a marker of cell proliferation, and anti-SMC- α actin. Ki-67-positive and SMC actin-positive cells in the media and neointima were counted from merged images

Figure 4. Modulation of IL-19 expression influences neointimal hyperplasia. Adenoviral delivery of IL-19 to balloon angioplasty-injured rat carotid arteries 1×10^8 AdLacZ, AdIL-19, or phosphate-buffered saline (vehicle), 15 minutes dwell time. **A–C:** Representative sections harvested 14 days after injury stained with H&E. **D–F:** Representative sections harvested 7 days after injury immunostained with anti-IL-19 antibody. Red-brown indicates positive immunoreactivity. **G:** IL-19 Western blot of arteries excised from rats treated as described. Morphological and statistical analysis of effect of modulation of IL-19 expression on neointima formation. **H:** Neointimal area; **I:** intimal/medial ratio; **J:** lumen area. Data shown are means $\pm$ SEM. Significance versus AdLacZ determined by analysis of variance ($n = 6$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Scale bars: $150 \mu\text{m}$ (**A–C**); $50 \mu\text{m}$ (**D–F**). Original magnifications: $\times 40$ (**A–C**); $\times 600$ (**D–F**).

from at least four areas of the artery per section, and four representative sections at least 100 to $150 \mu\text{m}$ apart from each other per carotid artery. Ki-67 and SMC- α actin-negative cells were excluded. Figure 5 shows a significant decrease in Ki-67-stained SMCs in AdIL-19-treated carotid arteries compared with AdLacZ and vehicle (5.6 ± 0.9 versus 12.9 ± 1.5 , and 14.2 ± 1.2 ; $P < 0.001$). Together, these data indicate that IL-19 expression can diminish intimal hyperplasia, likely by diminishing VSMC proliferation.

IL-19 Can Activate STAT3-Inducible Gene Expression

Many interleukins can stimulate STAT3 activation in inflammatory cells,¹⁶ and we hypothesized the same for IL-19 in VSMCs. Figure 6, A and B, shows that IL-19 can stimulate rapid and transient STAT3 activation, as determined by phosphorylation. STAT3 also translocated from the cytoplasm to the nucleus on IL-19 treatment (Figure 6C). Phosphorylated STATs dimerize and then translo-

Figure 5. Modulation of IL-19 expression influences neointimal VSMC proliferation. **A:** Ki-67-positive (red nuclei), SMC- α actin-positive (green cytoplasm) VSMCs in the media and neointima were counted from merged images from at least four representative stained tissue sections at least 100 to 150 μ m apart per carotid artery, from three different rats. **B:** Data shown are means $\pm$ SEM. Significance versus AdLacZ and PBS determined by analysis of variance ($n = 3$). *** $P < 0.001$. Scale bar = 50 μ m. Original magnifications: $\times 400$.

cate into the nucleus where they recognize specific DNA sequences or other transcription factors to influence target gene transcription.¹⁷ To identify gene expression induced by IL-19, which is known to be STAT3 inducible, cultured VSMCs were serum-starved to approximate quiescence, stimulated by IL-19 for 16 hours, and gene expression determined by biased cDNA array representing known downstream effectors of JAK/STAT signaling. In this screen, there were few detectable mRNA changes. However, Figure 6D is a Western blot showing that expression of an important regulatory protein, the signal transduction protein SOCS5 (suppressor of cytokine signaling 5), is rapidly and transiently increased in response to IL-19 treatment of VSMCs. SOCS5 is a member of a family of six proteins that are often synthesized *de novo* in response to cytokine stimulation and in many cases, transcriptional activation of SOCS family genes are mediated by the STAT proteins^{16,18}. No other SOCS protein mRNA was detectable on the array. This is the first description of expression of SOCS5 in VSMCs, and induction of SOCS5 by IL-19 in any cell type, and indicates that SOCS5 is an IL-19-responsive gene in human VSMCs.

Figure 6. IL-19 induces STAT3 phosphorylation. **A:** Representative immunoblot of human VSMCs that were serum-starved and then stimulated with 120 nmol/L recombinant IL-19 for the indicated times. Extracts were blotted with anti-phospho STAT3 (Tyr705), or total STAT3. **B:** Densitometry was performed and values normalized to total protein from at least three experiments. Asterisk indicates significant difference from unstimulated ($P < 0.05$). **C:** For STAT3 translocation, VSMCs were serum-starved for 24 hours and then stimulated with IL-19 for the times indicated followed by analysis of compartmental lysates by immunoblot. **D:** IL-19 induces SOCS5 protein expression. Representative immunoblot of human VSMCs that were serum-starved and then stimulated with IL-19 for the indicated times. Extracts were blotted with SOCS5 antibody. Anti-GAPDH was used as a loading control.

Suppressive Effects of IL-19 on Activation of MAPK Signal Transduction

Considering IL-19 effects on SOCS5 expression, we next hypothesized if IL-19 could reduce or inhibit signal transduction protein activation. For these experiments, cultured, human coronary artery VSMCs were serum-starved for 48 hours, pretreated with IL-19 for 8 hours, and then challenged with 10% FCS for 15 minutes. FCS was used because it contains multiple cytokines and growth factors. Results presented in Figure 7, A and B, show that IL-19 pretreatment can significantly reduce p44/42 MAPK activation by 59% (1500 ± 153 versus 3670 ± 328 for IL-19-pretreated and control-stimulated cells, respectively) ($P < 0.01$). Similarly, Figure 7, A and B, also shows IL-19 pretreatment can significantly reduce p38 phosphorylation by 56% (831 ± 377 versus 1915 ± 234 for IL-19-pretreated and control-stimulated cells, respectively; $P = 0.03$). This is not because of down-regulation of these kinases, as total levels of these proteins remains constant. No inhibition of Akt, p70S6K, or PAK kinase was observed. This is not because of IL-19 desensitization of either MAPK by prolonged IL-19 exposure because IL-19 alone does not activate either kinase (data not shown). Together, these data

Figure 7. IL-19 decreases MAPK activation. **A:** Representative immunoblot of human coronary artery VSMCs that were serum-starved and then pretreated with IL-19 for 8 hours. VSMCs were then stimulated with 10% FCS for 15 minutes, and extracts blotted with anti-phospho p44/42 or p38, and total p44/42 or p38. Anti-GAPDH was used as a loading control. **B:** Densitometry was performed and values normalized to total protein. Values and means from at least three experiments. **Asterisk** indicates significant difference from control ($P < 0.05$). Figures are representative of at least four performed. **C:** SOCS5 interacts with MAPKs. Immunoblot of human coronary artery VSMCs that were serum-starved, pretreated with IL-19 for 8 hours to induce endogenous SOCS5 expression, and then PDGF 15 minutes to induce phosphorylation of MAPKs. Both forward and reverse immunoprecipitation was performed on the same gel. Lysates were incubated with either SOCS5, p44/42, or p38 antibody, and immunoprecipitated. These antibodies used to immunoprecipitate are indicated above the blot. Immunoprecipitates were separated by SDS-PAGE in an individual lane on the same gel. The membrane was cut so that proteins of different molecular weight could be immunoblotted from the same gel, and immunoblotted with either SOCS5, p44/42, or p38 antibody, which are indicated under the blot. The input consisted of 10% of the IP lysate volume run in separate lanes on the same gel, and served as a positive control for the antibody. Representative of at least three experiments.

suggest that IL-19 has suppressive effects on MAPK signal transduction.

No inhibition of signal transduction was seen with less than 8 hours of IL-19 preincubation, suggesting that synthesis of an IL-19-inducible factor was necessary for IL-19 suppressive effects. SOCS proteins mediate their inhibitory effects by interaction with phosphorylated signaling intermediates and receptor chains, often resulting in a reduction in signaling.¹⁹ Based on its amino acid sequence, SOCS5 contains three potential MAPK family interaction domains (R/KXXXX#X#), where # is a hydrophobic residue.²⁰ Based on this, we hypothesized that the IL-19 inducible factor was SOCS5. To test this hypothesis and determine a potential mechanism for these inhibitory effects, VSMCs were stimulated with IL-19 to induce endogenous SOCS5 protein expression, and stimulated

with PDGF for 15 minutes to activate MAPK. Specific antibody for either SOCS5, p44/42, or p38 added to lysates, and these proteins were immunoprecipitated. Immunoprecipitates were separated by SDS-PAGE, and co-immunoprecipitated proteins immunoblotted with either SOCS5, p44/42, or p38 antibody. Figure 7C shows that SOCS5 interacts with both p44/42, and p38 in IL-19-stimulated VSMCs. These data strongly suggest that SOCS5 interaction with signaling proteins is a potential mechanism whereby IL-19 can inhibit inflammation-induced signal transduction.

Discussion

IL-19 is a recently described member of the IL-10 family of cytokines, and like IL-10 is considered to have anti-inflammatory effects in that it can induce lymphocytes to shift from a Th1 to a Th2 phenotype.⁶⁻⁸ In the present study we show that IL-19 is not normally expressed in unstimulated VSMCs, but can be induced by inflammatory cytokines and inflammatory stimuli. This is unexpected in that IL-19 expression had been ascribed to be restricted to immune cells. In our hands, IL-10 mRNA and protein are not detectable in quiescent or stimulated VSMCs, suggesting that in human VSMCs, IL-19 is not simply an IL-10 homologue. Immunohistochemical analysis demonstrated that IL-19 can be detected in medial and neointimal VSMCs in human arteries with transplantation vasculopathy, but not normal arteries, suggesting that IL-19 is an inflammation responsive cytokine in injured arterial VSMCs.

The mechanisms of inhibition of cellular activation by immunomodulatory cytokines are diverse and almost exclusively performed in immune cells, but nothing has been reported on the effects of IL-19 on VSMC pathophysiology. Anti-proliferative effects of IL-19 on a cancer cell line have been reported,²¹ and we found that addition of recombinant IL-19 to VSMCs resulted in a dose-dependent, anti-proliferative effect, as did infection of VSMCs with AdIL-19.

Together, these data confirm anti-proliferative effects of IL-19 on VSMCs. One study has reported anti-proliferative effects of IL-10 on VSMCs, and intraperitoneal injection of IL-10 decreased balloon angioplasty-induced intimal hyperplasia in rats.¹⁵ However, because IL-10 concentrations were systemic, the contribution of IL-10 to immune attenuation with subsequent reduction in intimal hyperplasia cannot be ruled out. It has been proposed that the observed growth inhibitory effects of IL-10 are because of inhibition of NF- κ B and NF- κ B-dependent inflammatory gene expression. We did not observe any inhibition of NF- κ B activation in cultured VSMCs (not shown). This is an interesting distinction that contrasts the anti-proliferative mechanism of IL-19 from IL-10. IL-19 is expressed in medial VSMCs as early as 1 day after injury, and neointimal cells of rat carotid arteries 7 days after balloon angioplasty. However, adenoviral overexpression of IL-19 directly to angioplasty-injured carotid arteries significantly decreased VSMC proliferation and development of neointimal

hyperplasia compared with controls. Because IL-19 is reported to be anti-inflammatory, we anticipated less leukocyte infiltration in the IL-19-expressing arteries compared with the two control groups of animals. We performed immunohistochemistry using antibody to lymphocytes and macrophages. In all groups, we observed a very low number of leukocytes that localized to the endothelial cell surface. However, the rat carotid artery balloon angioplasty model is generally proliferative rather than inflammatory in nature,^{13,14} and the numbers of leukocytes we observed in IL-19-infected groups as well as control groups were equally very low, and we were unable to generate statistical significance. A different model of vasculopathy in which inflammation was preeminent could be used for future studies. In this model, these data suggest that IL-19 expression can exert direct suppressive effects to VSMCs *in vivo* and have protective effects on the vascular response to injury.

The effector function of many interleukins are elaborated by activation of intracellular signaling cascades involving the JAK and STAT family of signaling proteins. IL-10 anti-inflammatory signaling in monocytes and T lymphocytes is known to signal through activation of STAT1 and STAT3,^{16,22} and one study using a breast cancer cell line shows that STAT3 translocates to the nucleus in response to IL-19.²¹ Tyrosine phosphorylated STATs dimerize and then translocate into the nucleus where they recognize specific DNA sequences or other transcription factors to influence target gene transcription. We found that IL-19 rapidly activated STAT3 activity, as assayed by tyrosine phosphorylation, and STAT3 nuclear translocation. With this as a starting point, we ascertained the effects of IL-19 stimulation on gene expression by cDNA array biased to known downstream effectors of JAK/STAT signaling. There were surprisingly few detectable mRNA changes. However, one that we were able to verify at the protein level was SOCS5, which we found to be rapidly and transiently induced in VSMCs by IL-19. No relationship has been reported between STAT3 activation and SOCS5 expression, and this is the first description of induction of this protein by IL-19, in any cell type. There are six SOCS family members that exert their inhibitory effects by binding to tyrosine phosphorylated residues on signaling intermediates, protein kinases, and receptor chains, resulting in an attenuation of signaling. Most of the literature focuses on SOCS1 through SOCS3, which in lymphocytes, are inducible by IL-10.¹⁹ Increased SOCS1 and SOCS3 have been identified in a mouse model of atherosclerosis.²³ Very little is known about SOCS4, SOCS5, and SOCS6, although SOCS5 is known to inhibit IL-6 signaling.²⁴ SOCS5 induction by IL-19 is noteworthy in that one mechanism by which negative regulation of cytokine-activation is mediated is through the action of the SOCS family proteins. SOCS family proteins have been reported to interact with and inhibit JAK proteins and growth factor receptors, and overexpressed SOCS1 and SOCS6 proteins inhibit insulin-dependent activation of p44/42, although direct interaction has not been shown.²⁵ In many cases, transcriptional activation of SOCS family genes are mediated by the STAT proteins themselves.^{18,24} Because SOCS proteins are reported to be *de novo* synthesized as a consequence of STAT activation, these proteins are considered to function as integral parts of a classical autocrine-negative autoreg-

ulatory feedback inhibitor of cytokine signaling. The rapid and transient nature of IL-19 induced SOCS5 expression might account for the observation that the greatest inhibitory effects of IL-19 are at earlier times after addition. We propose that IL-19 induced, STAT3 protein-mediated SOCS5 expression is one likely mechanism whereby IL-19 might exert its anti-proliferative and anti-restenotic effects.

Considering the known functions of the SOCS proteins in the context of IL-19 growth-suppressive effects, we hypothesized that IL-19 could reduce or inhibit signal transduction protein activation. Because SOCS5 contains three potential MAPK family interaction domains, we focused on p44/42 and p38 MAPK as potential targets of IL-19 suppressive effects. These kinases are also relevant in that they are demonstrated integrators of inflammation-inducible signaling, and both of these kinases have been shown to mediate VSMC activation and contribute to the vascular response to injury.^{26,27} Pretreatment of VSMCs with IL-19 results in a significant reduction in p44/42 and p38 MAPK activation. It was interesting in that no inhibition of several other non-MAPK family kinases was observed.

There were three reasons to suspect SOCS5 as a direct mediator of inhibition of these MAP kinases. First, it took at least 8 hours of pretreatment to induce these suppressive effects, suggesting that *de novo* synthesis of an intermediate was necessary; second, amino acid motif analysis indicates that SOCS5 contains three putative MAPK binding domains; and third, the known functions of SOCS family members as negative feedback integrators of cytokine signaling. We show that SOCS5 does directly interact with activated p44/42 and p38 in human VSMCs, and this likely constitutes at least one mechanism of IL-19 inhibition of signaling. This is the first report of SOCS5 interaction with these kinases. Based on its amino acid sequence, the potential does exist for binding to multiple MAP kinases in addition to p44/42 and p38. Other MAPK family members as targets of IL-19-induced SOCS5-mediated inhibition might also be likely.

No studies of IL-19 expression or function in vascular cell pathobiology have been reported. In addition to the surprising finding that IL-19 is expressed and growth suppressive in VSMCs, very little has been reported on the biology and function of SOCS5 in any cell type, and nothing at all in VSMCs. No relationship has been reported between STAT3 activation and SOCS5 expression in any cell type. Based on these data, our working hypothesis is that IL-19 induces suppressive effects on VSMC pathophysiological processes by STAT3-mediated SOCS5 expression. SOCS5 binds to and attenuates the activation of p44/42 and p38 MAPK. Our observations regarding IL-19 expression by VSMCs are novel in at least two broad aspects. The first is that the expression of this immunosuppressive cytokine has direct suppressive effects on VSMC activation and can modulate the VSMC response to injury *in vivo*. The second is the possibility that expression of this cytokine by inflamed vasculature, specifically VSMCs, may function as an autocrine-negative feedback to diminish the vascular response to injury.

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