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Original article

Interleukin-19 increases angiogenesis in ischemic hind limbs by direct effects on both endothelial cells and macrophage polarization

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ABSTRACT

Hypoxia in ischemic limbs typically initiates angiogenic and inflammatory factors to promote angiogenesis in attempt to restore perfusion. There is a gap in our knowledge concerning the role of anti-inflammatory interleukins in angiogenesis, macrophage polarization, and endothelial cell activation. Interleukin-19 is a unique anti-inflammatory Th2 cytokine that promotes angiogenic effects in cultured endothelial cells (EC); the purpose of this study was to characterize a role for IL-19 in restoration of blood flow in hind-limb ischemia, and define potential mechanisms. Hind limb ischemia was induced by femoral artery ligation, and perfusion quantitated using Laser Doppler Perfusion Imaging (LDPI). Wild type mice which received i.p. injections of rIL-19 (10 ng/g/day) showed significantly increased levels of perfusion compared to PBS controls. LDPI values were significantly decreased in IL-19$^{-/-}$ mice when compared to wild type mice. IL-19$^{-/-}$ mice injected with rIL-19 had significantly increased LDPI compared with PBS control mice. Significantly increased capillary density was quantitated in rIL-19 treated mice, and significantly less capillary density in IL-19$^{-/-}$ mice. Multiple cell types participate in IL-19 induced angiogenesis. IL-19 treatment of human microvascular EC induced expression of angiogenic cytokines. M2 macrophage marker and VEGF-A expression were significantly increased in macrophage and the spleen from rIL-19 injected mice, and M1 marker expression was significantly increased in the spleen from IL-19$^{-/-}$ compared with controls. Plasma VEGF-A levels are higher in rIL-19 injected mice. IL-19 decreased the expression of anti-angiogenic IL-12 in the spleen and macrophage. This study is the first to implicate IL-19 as a novel pro-angiogenic interleukin and suggests therapeutic potential for this cytokine.

1. Introduction

Peripheral artery disease (PAD) is often associated with diabetes and coronary artery disease, leading to significant morbidity (amputation) and mortality (myocardial infarction) in patients. Identification and characterization of molecules which not only limit tissue inflammation but also increase capillary density, collateral formation and perfusion have the potential to salvage ischemic tissue and can lead to new therapies for tissue repair and neovascularization. Hypoxia in ischemic limbs typically initiates angiogenic and inflammatory factors to promote angiogenesis in attempt to restore perfusion, and accordingly ischemic revascularization is a complex process involving multiple processes and cell types. While neovascularization and inflammation are independent biological processes, they are linked in response to injury and ischemia, and both inflammatory and anti-inflammatory cytokines participate in these processes. Endothelial cell (EC) paracrine and autocrine stimulation can result in migration and proliferation, and are an essential component of normal and pathophysiological processes including wound healing and angiogenesis [1–3]. In addition to well characterized angiogenic cytokines like VEGF, FGF, and CXCL1, it is accepted that many pro-inflammatory cytokines such as IL-1β, IL-6, IL-8, and IL-18 increase EC migration, proliferation, tube formation, and increased vascularity in vivo [4–6]. One exception is Interleukin-12, which is both pro-inflammatory and potently anti-angiogenic [7]. On the other hand, the role of and direct effects of anti-inflammatory interleukins on EC in initiation of angiogenesis are less clear. The prototypical anti-inflammatory cytokine, IL-10, has anti-angiogenic activity and is associated with VEGF down regulation, reduction of FGF and VEGF induced proliferation of microvascular EC [8]. Similarly, IL-4 can inhibit VEGF production and reduce vascularization, but can also induce migration and tube like structure formation in EC, activities consistent with angiogenesis [9–11]. IL-13 attenuates EC tube formation, and IL-20 has both pro- and anti-angiogenic effects [12–15]. Macrophages also participate in angiogenesis as the M2, or alternatively activated macrophages express several pro-angiogenic cytokines and thus must be included in any discussion of angiogenesis in vivo [16,17]. In summary, direct pro-angiogenic effects on EC, polarization of macrophage M2 phenotype, and inhibition of anti-angiogenic cytokines are all recognized pathways leading to angiogenesis; a modality which could reduce

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inflammation but not impair revascularization and would have obvious clinical benefits.

Interleukin-19 (IL-19) was discovered in 2001 [18], and is considered to be part of the IL-10 sub-family which includes IL-20, IL-22 and IL-24 [19,20]. IL-19 promotes an anti-inflammatory Th2 rather than the Th1 response in T-lymphocytes [21,22]. Unlike IL-10, IL-19 expression and activity are not restricted to leukocytes and is rather unique among interleukins. For example, neither IL-10, IL-4, nor IL-33 is expressed by EC or vascular smooth muscle cells (VSMC), precluding potential autocrine effects of these interleukins on the vasculature [23]. Little is reported regarding IL-19 effects on macrophage.

We recently reported that IL-19 was expressed in angiogenic tissue, and has potent pro-angiogenic effects on multiple human EC types, (umbilical vein, coronary artery, and microvascular) [24]. This manuscript reported that IL-19 is chemotactic and mitogenic for EC, promotes tube-like structure formation on Matrigel, and microvessel formation in the mouse aortic ring assay. These inaugural studies, though novel, were all cell culture or ex vivo based, and lacked validation in a relevant in vivo model of angiogenesis. Rodent hind limb ligation and ischemia is a well-established model for induction of neovascularization in vivo [25,26]. In the present study, multiple but complementary approaches were used to determine if IL-19 regulated neovascularization in the hind-limb ischemia model. In this manuscript we determined that in contrast to IL-10, IL-19 can increase perfusion in ischemic hind limbs and exerts its angiogenic effects by at least three mechanisms: direct effects on EC gene expression; local and systemic M2 macrophage polarization and VEGF-A expression, and suppression of IL-12 expression in macrophage. Together, this implicates IL-19 as a link for two major processes; anti-inflammation and angiogenesis, and could identify IL-19 as a previously unrecognized pro-angiogenic modality in treatment of PAD.

2. Materials and methods

2.1. Animals

Wild type C57BL/6 mice were purchased from Jackson Labs. IL-19 knockout mice were generated using the VelociGene method and IL-19$^{-/-}$ mice identified by genotyping of tail DNA by PCR using specific primers as we described [27]. Age and sex-matched male and female littermates were used for these studies. The hind limb ischemia model was performed as described [28]. Briefly, mice were anesthetized by injection of ketamine and xylazine, the femoral artery is dissected from the femoral vein, the artery occluded at two points using double knots, and the femoral artery between these knots excised. Ten ng/g/day recombinant murine IL-19 was administered i.p. 24 h post-surgery. Laser Doppler scanning was performed by an operator blinded to the identity of each mouse, immediately following surgery, and at days 3, 7, 10, and 15 days post-surgery using a Laser Doppler Imager (Moor Instruments, Cambridge, UK). After 15 days, mice were euthanized and gastrocnemius muscle was prepared for immunohistochemistry. Some mice were injected i.p. with 10 ng/g/day murine rIL-19 (eBioscience) or an equivalent volume of PBS five days per week for the duration of the study. All animal procedures followed Temple University-IACUC approved protocols.

2.2. Immunohistochemistry

Five-micrometer sections from paraffin embedded gastrocnemius muscle were blocked in 10% goat serum. Sections were incubated with primary antibody (anti-CD31 and anti-IL-19, from AbCam, Inc) at 1 μg/ml in 1% BSA/PBS and were applied for 1 h., followed by incubation with biotinylated secondary antibody (1:200), followed by avidin-biotin peroxidase complex each for 30 min as we have described [24,27]. Non-specific identical isotype control (Neomakers # NC-100-P, and Biolegend #400601) antibodies were used as negative controls. Macrophages were identified by immunostaining with anti-CD68 antibody (BioRad, Inc) and quantitated by manual counting of 9 randomly chosen HPFs from at least three different sections of ischemic hind limb of 6 animals per group 5 days post ligation surgery. For quantitation of capillary density, three transverse serial sections of gastrocnemius muscle spaced 100-200 μm apart were immunostained from at least 6 mice in each group. Capillaries (CD31 positive structures surrounding a lumen) were counted from three per high powered fields per section and reported per mm^2.

2.3. Human microvascular EC culture

Human microvascular endothelial cells (hmvEC) were obtained from Lonza, Inc. and cultured in growth media from the manufacturer as we described [24]. Cells from passage 2 to 4 were used. For gene expression studies, growth media were replaced with basal media supplemented with 1% fetal calf sera for 24 h, then stimulated with 100 ng/ml IL-19 for the times indicated.

2.4. Bone marrow derived macrophage

To generate BMDM, mice femurs and tibiae were flushed with sterile DMEM. After lysis of red blood cells, total BM cells were plated at a density of 3,5 × 10^6 cells per 10-cm Petri dish in 10 ml macrophage growth medium (complete DMEM medium with 10% FBS and 100 ng/ml M-CSF) (Peprotech Inc.) and were allowed to differentiate for 5–7 days. Cells were fed with additional 5 ml of growth medium on day 3. On day 7, cells were lifted with Versene 1× solution (GIBCO) at 37 °C and were re-plated in 12- or 6-well plates (1 × 10^6 cells per ml per well (12-well) or 2 × 10^6 cells per 3 ml per well (6-well)) in macrophage complete media (DMEM + 10%FBS).

2.5. RNA extraction and quantitative RT-PCR

RNA from cultured cells, hind limb, or spleen was isolated and reverse transcribed into cDNA as we have described, and target genes were amplified using an Eppendorf Realplex4 Mastercycler [27,29]. Multiple mRNAs (Ct values) were quantitated simultaneously by the Eppendorf software. Gene expression in IL-19-stimulated cultured human mvEC was performed using the human angiogenesis RT^2 Profiler PCR array from SABiosciences as described by the manufacturer. Primer pairs were purchased from Integrated DNA Technologies, (Coralville, IA), SYBR green used for detection. The following primer pairs were used:

Mouse GAPDH: F: GCAAGGACACTGAGCAAGAG, R: GGGTCTGGGATG GAAATTGT,
Mouse Arginase 1:F: AAGAATGGAAGAGTCAGTGTGG, R: GGGAGTGT TGATGTCAGTGTG
Mouse Arginase 2: F: CAGAAGGTGATGGAACAGACA, R: GCCAGTTT AGGGTCAAATGC
Mouse Ym1: F: AGAGTGCTGATCTCAATGTGG, R: GGGCACCAATTCCA GTCTTAG
Mouse KLF4: F: ACTTGTGACTATGCAGGCTG, R: ACAGTGGTAAGGTT TCTCGC
Mouse VEGF-A: F: GGCAGCTTGAGTTAAACGAAC, R: TGGTGACATG GTTAATCGGTC
Mouse IL-12p40: F: GTGAAGCACCAAATTACTCCG, R: AGAGACGCCA TTCCACATG
Human GAPDH: F: CGAGAGTCAGCCGCATCTT, R: CCCCATGGTGTCTG AGCG,
Human IL-8: F: CCAGGAAGAAACCACCGGA, R: GAAATCAGGAAGGC TGCCAAG
Human HGF: F: ATCAAATGTCAGCCCTGGAG, R:CCTCTGGATTGCTT GTGAAAC
Human CXCL1: F: TGCTCCTGCTCCTGGTAG, R: CTTCTGGTCAGTTGGA TTTGTC