IL-9 Induces VEGF Secretion from Human Mast Cells and IL-9/IL-9 Receptor Genes Are Overexpressed in Atopic Dermatitis

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Abstract

Interleukin 9 (IL-9) has been implicated in mast cell-related inflammatory diseases, such as asthma, where vascular endothelial growth factor (VEGF) is involved. Here we report that IL-9 (10–20 ng/ml) induces gene expression and secretion of VEGF from human LAD2. IL-9 does not induce mast cell degranulation or the release of other mediators (IL-1, IL-8, or TNF). VEGF production in response to IL-9 involves STAT-3 activation. The effect is inhibited (about 80%) by the STAT-3 inhibitor, Stattic. Gene-expression of IL-9 and IL-9 receptor is significantly increased in lesional skin areas of atopic dermatitis (AD) patients as compared to normal control skin, while serum IL-9 is not different from controls. These results imply that functional interactions between IL-9 and mast cells leading to VEGF release contribute to the initiation/propagation of the pathogenesis of AD, a skin inflammatory disease.

Introduction

IL-9 was first described in the late 1980s as a member of a growing number of cytokines that has pleiotropic functions in the immune system [1]. IL-9 was initially purified and characterized as a T cell and mast cell growth factor [2]. IL-9 production was first associated with the Th2 phenotype, and many of the preliminary functions of IL-9 were tested in models of Th2-associated immunity [3]. Th17 cells, which are defined by high levels of sensitization to foods [21]. Many patients with moderate atopic dermatitis (AD) were shown to have high levels of sensitization to foods [21]. IL-9 could be involved in the pathogenesis of inflammatory skin disorders, such as AD, characterized by chronic skin inflammation that also involves mast cells [22]. Here we show that IL-9 gene expression is increased in lesional AD skin and stimulates VEGF release from cultured mast cells.

Materials and Methods

Reagents

Human IL-9 was purchased from Sigma (St. Louis, MO). STAT3 inhibitor Stattic was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Citation: Sismanopoulos N, Delivanis DA, Alysandratos KD, Angelidou A, Vasiadi M, et al. (2012) IL-9 Induces VEGF Secretion from Human Mast Cells and IL-9/IL-9 Receptor Genes Are Overexpressed in Atopic Dermatitis. PLoS ONE 7(3): e33271. doi:10.1371/journal.pone.0033271

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Funding: This work was supported in part from National Institutes of Health grant AR47652 awarded to TCT. No additional external funding received for this study. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Received January 11, 2012; Accepted February 9, 2012; Published March 8, 2012

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PLoS ONE | www.plosone.org 1 March 2012 | Volume 7 | Issue 3 | e33271
Culture of human mast cells

LAD2 mast cells (kindly supplied by Dr. A.S. Kirshenbaum, National Institutes of Health, Bethesda, MD), derived from a human mast cell leukemia [23], were cultured in StemPro-34 medium (Invitrogen, Carlsbad, CA) supplemented with 100 U/ml penicillin/streptomycin and 100 ng/ml recombinant human stem cell factor (rhSCF, Stemgen, kindly supplied by Swedish Orphan Biovitrum AB, Stockholm, Sweden). Cells were maintained at 37°C in a humidified incubator at 5% CO2.

VEGF release assay

LAD2 cells (1×105 cells/250 μl) were distributed in 96-well microtiter assay plates in triplicate and stimulated in complete culture medium with the indicated concentrations of IL-9. VEGF was determined in cell-free supernatants with a commercial ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturer’s directions. VEGF secretion data are expressed as pg/106 cells. For inhibition studies, inhibitors were added to the media 30 min prior to stimulation.

ELISA analysis of STAT3 phosphorylation

LAD2 cells were plated in 24-well plates (3×103 cells/well) in complete media. Cells were stimulated with IL-9 for the indicated time-points. Stimulation was terminated by the addition of ice-cold PBS. Cells were washed once with PBS and then lysed in cell lysis buffer ( #9803s, Cell Signaling Danvers, MA) and sonicated briefly. Equal amounts of protein from the cell lysates were used. Phospho-STAT3 levels were determined in the cell lysates with a commercial Elisa kit (Cell Signaling) according to the manufacturer’s directions.

Patients and biopsies

Full depth (3 mm3) punch skin biopsies were collected from subjects (patients and controls) who had not received any medication for 15 days prior to the biopsy and were seen at the 2nd Department of Dermatology of the Attikon General Hospital, Athens University Medical School, Athens, Greece. The Medical Ethics Committee of Attikon Hospital Institution’s Human Investigation Review Board (HIRB) approved this protocol. All participants gave their written informed consent according to the Declaration of Helsinki Principles. Patients were free from any other medical problems. All biopsies (patients and controls) were obtained from non-exposed skin (back and gluteal) and were immediately placed in RNAlater solution (Ambion, Inc., Austin, Texas, USA) and stored at −20°C.

PCR and quantitative PCR

Total RNA from skin biopsies or cultured mast cells was isolated using Trizol (Invitrogen), according to the manufacturer’s instructions. Reverse transcription was performed with 200 ng of total RNA using the iScript cDNA synthesis kit (Ambion, Austin, TX).

In order to measure IL-9, and IL-9 receptor (IL-9r) gene expression, quantitative real time PCR was performed using Taqman gene expression assays. The following probes obtained from Applied Biosystems, were used: IL-9, (ID: Hs00914237_m1); IL-9 receptor (ID: Hs01106522_m1); Human GAPD (GAPDH) Endogenous Control (VIC/TAMRA Probe, Primer Limited), (Number: 4310884E). The cycling conditions consisted of 1 cycle of 50°C for 2 min, 1 cycle of 95°C for 10 min, 40 cycles of 95°C for 15 s, 1 cycle of 60°C for 1 min, 1 cycle of 95°C for 15 s, 1 cycle of 60°C of 30 sec and 1 cycle of 95 for 15 s. Relative mRNA abundance was determined from standard curves run with each experiment, and IL-9, and IL-9 receptor expression was normalized to GAPDH endogenous control.

Results

IL-9 stimulates VEGF production in human mast cells

To examine the effect of IL-9 on VEGF secretion, LAD2 cells were treated with IL-9 (10, 20 ng/ml) for 48 hr. VEGF mRNA (measured at 6 hrs) was increased after IL-9 stimulation (Fig. 1A). IL-9 also stimulated release of VEGF with a maximum of 860 pg/106 cells at 10 ng/ml, a 2-fold induction (Fig. 1B). There was no apparent difference between 10 and 20 ng/ml IL-9 and lower concentrations did not have any effect. There was no degranulation as measured by beta-hexosaminidase release or release of IL-1, IL-8 or TNF (results not shown).

STAT3 Phosphorylation and activation is involved in IL-9-induced VEGF release

We investigated downstream events associated with stimulus-receptor coupling. Stimulation with IL-9 (10 ng/ml) for 5,10, or 20 min, increased STAT3 phosphorylation, which was detected within 5 min (Fig. 2A). Pretreatment with the inhibitor of the

Figure 1. IL-9 stimulates VEGF production in human mast cells. (A) Gene expression. LAD2 cells were stimulated with IL-9 for 6 hrs, RNA was extracted and relative VEGF mRNA levels were determined by real-time PCR. (B) Protein release. LAD2 cells were stimulated with the indicated concentration of IL-9 (10–20 ng/ml) for 48 hrs. VEGF was measured in the supernatant fluid by ELISA. Data are the mean ± SD of 3 separate experiments performed in triplicate (*P<0.05 versus unstimulated cells).

doi:10.1371/journal.pone.0033271.g001
STAT3 pathway. Stattic, blocked IL-9-induced VEGF release without significantly affecting basal release (Fig. 2B). Together, these data demonstrate that IL-9 induces phosphorylation of STAT3 in human mast cells and activation of STAT3 is necessary for VEGF production.

IL-9 and IL-9 receptor mRNA expression is increased in skin of patients with AD

Analysis of skin biopsies from AD lesional (n = 12) and control normal skin (n = 16) showed that IL-9 (Fig. 3A) and IL-9r (Fig. 3B) gene expression is significantly higher in lesional AD skin than normal control skin.

IL-9 levels in serum of patients with AD

Serum levels of IL-9 were not different in AD patients as compared to controls (Fig. 4).

Discussion

We report here for the first time that IL-9 induces VEGF release from human mast cells. This effect is greater than what has been previously reported for IL-33 [24]. IL-9 cannot stimulate human mast cell degranulation on its own or the production of other cytokines like IL-1, TNF, or IL-8. The amount of IL-9 inducing maximal VEGF release was 10 ng/ml, while 0.1 and 1 ng/ml had no effect (results not shown). The concentration we used (10 ng/ml) may be far higher than the concentrations needed for responses in some IL-9 dependent cell lines, but less than the dose (20 ng/ml) used for differentiation of Th17 cells [5], and lower than the dose (30 ng/ml) shown to stimulate cytokine gene expression from rodent mast cells [25]. The combination of SP and IL-9 did not produce any higher release than either trigger alone (results not shown). Here we also show for the first time that gene expression of IL-9 and IL-9 receptor are increased in lesional AD skin. In contrast, IL-9 was not increased in AD patients. A recent paper investigating serum IL-9 levels in systemic sclerosis, where it was elevated, also reported no difference between AD levels and controls [26].

IL-9 is a cytokine produced especially by CD4+ T-cells, but it appears that mast cells are also capable of producing IL-9 [7]. IL-9 is mostly expressed on mast cells [27], and also by regulatory T (TReg) cells, T helper 17 (TH17) cells and antigen-presenting cells.
of heparin binding growth factors and is a major pro-angiogenic factor involved in many inflammatory diseases [33]. The progression of inflammation parallels the dermal angiogenesis in murine models of atopic dermatitis [31]. The VEGF 121 isoform also causes vascular permeability [36,37]. Mast cells can secrete VEGF in response to IgE [30], substance P (SP) [24], and corticotropin-releasing hormone (CRH) [39], secreted under stress.

Mast cells are found in large numbers in around blood vessels in the skin where they participate in allergic and inflammatory reactions through release of multiple mediators with potent vasodilatory, inflammatory and nociceptive properties [40]. In addition to VEGF, histamine increases vascular permeability [41] and stimulates cutaneous sensory nerves [42] contributing to pruritus. In acute AD lesions, mast cells are normal in number but they appear to be degranulated [43]. In chronic lesions, however, especially in areas of lymphocytic infiltration in the papillary dermis, mast cells numbers are significantly increased, in close association with endothelial cells [44,45]. Mast cell activation levels were shown to be correlated with the severity of AD [46]. Skin mast cells may have important functions as “sensors” of environmental and emotional stress [47].

The present results is the first indication that interactions between IL-9 and mast cells may be important in inflammatory skin diseases [48] where there is increased angiogenesis, such as AD. They may also represent novel therapeutic targets.

Statistical analysis
Data are expressed as the mean ± SD. Statistical significance between experimental samples and controls was calculated using the Student’s t-test. P<0.05 was considered statistically significant.

Acknowledgments
We thank Swedish Orphan Biovitrum AB, (Stockholm, Sweden) for their kind gift of rhSCF, Stengen and Drs. A. S. Kirshenbaum and Dean Metcalfe (NIH) for the LAD2 mast cells.

Author Contributions
Conceived and designed the experiments: NS TCT. Performed the experiments: NS DAD KDA AA. Analyzed the data: NS TCT. Wrote the paper: NS TCT.

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