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Towards a role of interleukin-32 in atherosclerosis

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ABSTRACT

Background: IL-32 has been previously shown to promote inflammation in rheumatoid arthritis patients and to contribute to IL-1β-induced ICAM-1 as well as other proinflammatory cytokines synthesis in human umbilical endothelial cells (HUVECs). Given the high rate of atherosclerosis in RA, these observations suggest that IL-32 may be involved in the inflammatory pathways of atherosclerosis.
Methods: mRNA and protein levels of IL-32 were determined in human atherosclerotic arterial vessel wall tissue by quantitative real-time PCR and immunohistochemistry. HUVEC and M1/M2 macrophages were stimulated with proinflammatory cytokines and TLR ligands to assess IL-32 mRNA induction. Human THP1 macrophages were transduced with AdIL-32γ, to investigate induction of several proatherosclerotic mediators. Finally, aortas from IL-32γ transgenic mice were studied and compared with aortas from age-matched wild-type mice.
Results: IL-32 expression was detectable in human atherosclerotic arterial vessel wall, with the expression of IL-32β and IL-32γ mRNA significantly enhanced. TLR3-ligand Poly I:C in combination with IFNγ were the most potent inducers of IL-32 mRNA expression in both HUVEC and M1/M2 macrophages. Adenoviral overexpression of IL-32γ in human THP1 macrophages resulted in increased production of CCL2, sVCAM-1, MMP1, MMP9, and MMP13. The IL-32γ transgenic mice chow a normal fat diet exhibited vascular abnormalities resembling atherosclerosis.
Conclusions: IL-32 acts as a proinflammatory factor and may be implicated in the inflammatory cascade contributing to atherosclerosis. By promoting the synthesis of matrix metalloproteinases, it may further contribute to plaque instability. Further studies are warranted to investigate whether IL-32 may serve as a potential therapeutic target in fighting atherosclerosis.

1. Introduction

Cardiovascular diseases (CVD) are the most frequent cause of death among the population living in the developed countries. The development of an acute event is typically conditioned by the formation of atherosclerotic lesions that subsequently become unstable. Many studies have demonstrated that besides dyslipidemia, inflammation plays a crucial role in these processes [1]. Accordingly, elevation of the sensitive acute-phase proteins C-reactive protein and serum amyloid A protein predicts a poor outcome in patients with unstable angina and may reflect an important inflammatory component in the pathogenesis of this condition [2]. Moreover, inflammatory pathways involved in the pathogenesis of chronic inflammatory conditions such as rheumatoid arthritis (RA) have been suggested to accelerate atherosclerosis and explain the increased CV morbidity and mortality observed in these patients as compared with the general population [3].

Cytokines are key modulators of inflammatory processes. Previous studies have indicated that several proinflammatory cytokines including tumor necrosis factor alpha (TNFα), interleukin (IL)-1β, IL-6, IL-18 and interferon-gamma (IFNγ) contribute to the development of atherosclerosis [4–9]. Interleukin (IL)-8 (CXCL8) and adhesion molecules such as ICAM-1 and VCAM-1 contribute to

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the direct migration and facilitate the penetration of inflammatory cells into the intima layer of the vessel wall at sites of plaque formation [9,10]. Inflammation further promotes the differentiation of recruited monocytes into macrophage foam cells, and precipitates acute thrombotic complications of atheroma. Accordingly, IFNγ arising from the activated T lymphocytes in the plaque can halt collagen synthesis by smooth muscle cells (SMCs), promoting plaque instability [4,11]. Production of cytokines can be triggered by the stimulation of immune receptors such as toll-like receptors (TLRs). In line with this, stimulation of TLR3 prompts the production of IFNγ, whereas TLR4 and TLR2 stimulation result mainly in an increase of TNFα, IL-1β, IL-6 and IL-8 [12–14].

IL-32 is a novel proinflammatory cytokine, which promotes inflammation by inducing other proinflammatory cytokines including of TNFα, IL-1β, IL-6 and IL-8 [15–17]. An important role for IL-32 in the pathogenesis of rheumatoid arthritis has been recently proposed [18–20]. In addition, IL-32 appears to be a critical regulator of endothelial function, and is therefore likely to promote the process of atherosclerosis as it potentiates IL-1β-induced ICAM-1 and production of proinflammatory cytokines in endothelial cells [9]. The present study aims to provide more data sustaining a role of IL-32 in the pathogenesis of human atherosclerosis.

2. Materials and methods

2.1. Vascular tissue

Vascular specimens were isolated from atherosclerosis patients undergoing carotid endarterectomy and were used for determining IL-32 protein and mRNA expression by immunohistochemistry (IHC) and quantitative real-time PCR analysis, respectively. To asses normal IL-32 mRNA levels in vascular tissue, renal artery specimens without atherosclerotic lesions isolated from healthy kidney transplant donors were used. Informed consent and ethical approval of the medical ethics committee of the Radboud University Nijmegen Medical Centre were acquired.

2.2. IL-32 mRNA and protein expression

Vascular specimens were directly stored in liquid nitrogen until RNA isolation. RNA was extracted from the vascular specimens by using MagNa lyser green ceramic beads in a MagNa lyser apparatus (Roche, Basel, Switzerland) and the RNeasy mini Kit (Qiagen, Valencia, California, USA) was used to isolate RNA. RNA was transcribed into cDNA as described by Heinhuis et al. [18] and used for determining IL-32 mRNA expression by quantitative real-time PCR. IL-32 primers were designed with Primer Express 2.0 (Applied Biosystems, Foster City, CA, USA) and manufactured by Biolegio (Nijmegen, The Netherlands). For IHC analysis, vascular specimens were imbedded in paraffin as described by Joosten et al. [20] and stained for IL-32 using goat-anti-IL32 (AF3040) antibody from R&D Systems (Minneapolis, MN, USA).

2.3. Isolation of PBMCs and differentiation into M1 and M2 macrophages

Blood from healthy donors was used to isolate peripheral blood mononuclear cells (PBMCs) by density centrifugation diluted 1:1 in pyrogen-free saline over Ficoll-Paque (Pharmacia Biotech AB, Uppsala, Sweden). PBMCs were resuspended in RMPI-1640 (Gibco-Invitrogen, Paisley, UK) and seeded in 24-well culture plates (Corning Incorporated, Corning, NY, USA). Subsequently, PBMCs were incubated at 37 °C with 5% CO_2 for 45 min in an incubator for monocyte adherence. Non-adherent cells were washed away with phosphate-buffered saline (PBS) and RPMI-1640 medium containing glutamine, pyruvate, penicillin/streptomycin, 10% heat-inactivated human serum complemented with 100 ng/ml granulocyte–macrophage colony-stimulating factor (GM-CSF) for M1 or 100 ng/ml macrophage colony-stimulating factor (M-CSF) for M2 differentiation (R&D Systems, Minneapolis, MN, USA). After 3 days of differentiation, M1 and M2 macrophages were stimulated with different stimuli such as toll-like receptors (TLRs) ligands and cytokines.

2.4. Isolation of HUVEC

HUVEC were isolated from umbilical cords from healthy donors after obtaining informed consent. Cells were cultured in RMPI-1640 (Gibco-Invitrogen) supplemented with penicillin/streptomycin, glutamine, pyruvate, heat-inactivated pooled human serum (10%), and heat-inactivated FBS serum (10%). HUVEC were cultured in 0.2% w/v gelatinized (Sigma–Aldrich, St. Louis, MO, USA) tissue flasks/plates (Corning Incorporated) at 37 °C and 5% CO_2.

2.5. In vitro stimulation of M1/M2 macrophages and HUVEC

M1- or M2-differentiated macrophages and HUVEC were stimulated with 1 μg/ml Pam3Cys (EMC Microcollections, Tuebingen, Germany), 50 μg/ml Poly I:C (Invivogen, Toulouse, France), 100 ng/ml LPS (Sigma–Aldrich), 100 ng/ml TNFα (R&D Systems), 10 ng/ml IL-1β (R&D Systems), 100 ng/ml IFNγ (R&D Systems), or Poly I:C/IFNγ (50 μg/ml and 100 ng/ml respectively) in serum free RMPI-1640. Twenty-four hours post stimulation, total RNA was isolated by adding Tri-reagent (Sigma–Aldrich) to the cells and processed as described by Heinhuis et al. [18]. Finally, IL-32 mRNA expression was accessed by quantitative real-time PCR.

2.6. Adenoviral overexpression of IL-32γ in human THP1 cells

THP1 cells were transduced with AdControl or AdIL-32γ as described by Heinhuis et al. [19]. Transduced cells were incubated at 37 °C and 5% CO_2. Forty-eight hours later, supernatants were collected and used to determine CCL2, sVCAM-1, MMP1, MMP3, MMP9, and MMP13 secretion by ELISA (R&D Systems) or Luminex technology (Millipore, Billerica, MA, USA).

2.7. Aortic tissue from IL-32γ transgenic mice

Aortas were obtained from transgenic mice expressing human IL-32γ as described by Choi et al. [21]. Subsequently, aortic tissues were formalin-fixed paraffin-embedded and hematoxylin/eosin (H&E) stained. Eight month-old age-matched aortic tissue from Wild-Type (WT) mice were used as control. Both transgenic and WT mice were fed standard laboratory chow.

3. Results

3.1. IL-32 is abundantly present in human atherosclerotic vascular tissue

To investigate whether IL-32 is expressed in atherosclerotic vascular tissue, human arteria carotis specimens were used for determining IL-32 mRNA and protein expression. First, we used a quantitative real-time PCR primer set that detects all the IL-32 isoforms together in a single PCR. Secondly, four different quantitative real-time PCR primer sets were used to detect IL-32α, IL-32β, IL-32γ, and IL-32δ. Fig. 1A shows that total IL-32 expression is increased in atherosclerotic compared with healthy control tissue. Furthermore, IL-32β and IL-32γ were both significantly increased (Fig. 1A), whereas IL-32α and IL-32δ expression was not signifi-

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