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CXCL8 hyper-signaling in the aortic abdominal aneurysm☆

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ABSTRACT

There are indications for elevated CXCL8 levels in abdominal aortic aneurysm disease (AAA).

CXCL8 is concurrently involved in neutrophil-mediated inflammation and angiogenesis, two prominent and distinctive characteristics of AAA. As such we considered an evaluation of a role for CXCL8 in AAA progression relevant.

ELISA's, real time PCR and array analysis were used to explore CXCL8 signaling in AAA wall samples. A role for CXCL8 in AAA disease was tested through the oral CXCR1/2 antagonist DF2156A in the elastase model of AAA disease.

There is an extreme disparity in aortic wall CXCL8 content between AAA and aortic atherosclerotic disease (median [IQR] aortic wall CXCL8 content: 425 [141–1261] (AAA) vs. 23 [2.8–89] (atherosclerotic aorta) μg/g protein ($P < 1 \cdot 10^{-14}$)), and abundant expression of the CXCR1 and 2 receptors in AAA. Array analysis followed by pathway analysis showed that CXCL8 hyper-expression in AAA is followed increased by IL-8 signaling (Z-score for AAA vs. atherosclerotic control: 2.97, $p < 0.0001$).

Interference with CXCL8 signaling through DF2156A fully abrogated AAA formation and prevented matrix degradation in the murine elastase model of AAA disease ($p < 0.001$).

CXCL8-signaling is a prominent and distinctive feature of AAA, interference with the pathway constitutes a promising target for medical stabilization of AAA.

1. Introduction

An Abdominal Aortic Aneurysm (AAA) is a common pathology and a major cause of death due to rupture [1]. Most AAAs are asymptomatic and remain undetected until rupture [1]. Hence, some countries instigated nationwide screening programs for the identification of AAA. These programs resulted in a major increase in patients with an identified AAA, most of them small in size.

In accordance to prevailing guidelines these patients with smaller AAAs are kept under surveillance until the AAA reach the threshold for repair at 55 mm. It is estimated that up to 70% of the patients in the watch and follow up program will eventually reach the 55 mm intervention threshold [2]. Accordingly, it has been pointed out that pharmaceutical intervention reducing or inhibiting progression of small AAA, and thus reducing the need for surgical repair could have major advantages; both from a patients' as from a socio-economical perspective [3]. Despite clear preclinical successes, no pharmaceutical intervention has been proven to be effective so far [4].

The pathology of growing AAAs is thought to be a localized chronic inflammatory response that is accompanied and perpetuated by exaggerated angiogenesis and a proteolytic imbalance; the latter is being held responsible for a progressive weakening of the aortic wall [1]. The actual molecular basis has not been identified.

We previously documented CXCL8 hyper-expression as a clear distinctive and unique feature of AAA with 300-fold higher CXCL8 protein levels in the aneurysm wall than in advanced aortic atherosclerotic wall samples [5,6]. CXCL8 has comprehensive chemotactic effects on a wide-variety of immune cells, in particular *but* not-exclusively on

Abbreviations: AAA, abdominal aortic aneurysm

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neutrophils; a cell type that is explicitly implicated in AAA disease [5,7,8]. Moreover, CXCL8 stimulates protease expression and inflammation [9], and exerts strong pro-angiogenic effects by promoting chemotaxis and proliferation of endothelial cells [10–12].

In this context, we considered further examination of a putative role for CXCL8 signaling as a potential therapeutic target in AAA disease relevant. The present study confirms the CXCL8 hyper-expression and exaggerated activation of the CXCL8 downstream pathways in human aneurysms, and shows that interference with CXCL8 signaling through the oral CXCR1/2 antagonist DF2156A fully abrogates aneurysm formation in an accepted model of AAA disease (the murine elastase model).

2. Methods

2.1. Human samples

Collection and handling of the aneurysm and control aortic wall samples was performed in accord with the guidelines of the Medical and Ethical Committee Leiden University Medical Center, Leiden, The Netherlands, and the "code of conduct for responsible use" by the Dutch Federation of Biomedical Scientific Societies (https://www.federa.org/sites/default/files/digital_version_first_part_code_of_conduct_in_uk_2011_12092012.pdf) [13]. Plasma samples used were from the Aneurysm-Express biobank [14]. This study is approved by the Medical Ethics Committees of the participating hospitals, and all participants provided written informed consent.

We obtained tissue from anterior-lateral aneurysm wall during elective surgery for asymptomatic AAA (> 5.5 cm or larger). Aortic tissue samples removed along with the renal artery during kidney explantation from brain-dead, heart-beating, adult organ donor, were used as control samples. Aortic wall samples were divided in two parts. One half was immediately snap-frozen in CO2-cooled isopentane or liquid N2 and stored at −80 °C for later analysis. The other half was fixed in 4% formalin for 12 h and decalcified. The latter segments were paraffin embedded and 4 μm sections were processed into slices.

For immunohistochemistry, sections (n = 10 AAA, n = 10 control atherosclerotic aortic wall samples) were deparaffinized, treated for 10 min with H_2O_2 to block endogenous peroxidase activity, and incubated overnight at room temperature with the primary antibody diluted in PBS-1% albumin. The following primary antibodies were used: human myeloperoxidase (A398, DAKO, Amstelveen, The Netherlands), CXCL8 (bs-078012, Bioss, Huissen, The Netherlands), CXCR1 (ab124344, Abcam, Cambridge UK), CXCR2 (bs-1629R, Bioss), pERK1/2 (1481-1 Epitomics, Leiden, The Netherlands) and phospho-PKCΔ/ϕ (CST 9376S, Cell Signaling, Leiden, The Netherlands).

CXCL8 mRNA expression was quantified by semi quantitative RT, to that end a total RNA extraction was performed according to manufacturer's instructions. cDNA was prepared by using a Promega kit (Promega, Leiden, the Netherlands) for RT-PCR. For the determination of mRNA expression we used an established CXCL8 primer/probe set (Thermo Fisher Scientific, Bleiswijk, The Netherlands), the mastermix (Eurogentec, Maastricht, the Netherlands) and the ABI-7700 system (Thermo Fisher Scientific) as previously described [13]. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) (Thermo Fisher Scientific) was used for normalization.

Aortic wall CXCL8 protein content was determined using the Aneurysm-Express Biobank [14] (n = 238 AAA samples and n = 26 control atherosclerotic samples) via ELISA, employing Luminex multi-analyte profiling technology [15], using a bio-plex system (Bio-Rad, Veenendaal, the Netherlands). Total protein concentration of every sample was quantified via a BCA protein measurement method (Thermo Fisher Scientific). All measured concentrations were related to the protein concentrate of every sample. Inter-assay coefficient of variation was < 10%.

Microarrays: RNA extraction was performed from full thickness aortic wall samples from 31 AAA patients (mean age 69.5 yrs. mean diameter 62.3 ± 12.1 mm) collected during elective aneurysm repair and 9 control samples (infra renal aorta obtained during kidney procurement for donation).

RNA from aneurysm wall was labeled and hybridized to Illumina HumanHT-12 v4 BeadChips (Illumina, Eindhoven, the Netherlands). Arrays were scanned with an Illumina iScan microarray scanner. Bead level data preprocessing was done in Illumina GenomeStudio.

Analysis of array data: Quantile normalization and background reduction were performed according to standard procedures in the Illumina GenomeStudio software. Gene expression data have been deposited at Gene Expression Omnibus under the GEO Accession number GSE98278.

Association of genome-wide expression data with AAA phenotype revealed 11,486 transcripts with $P < 0.05$. These differentially expressed transcripts were used as an input for pathway analysis through Ingenuity Pathway Analysis suite (http://www.ingenuity.com, accessed 2016). Levels of significance were determined using Fisher's exact tests implemented in the software.[16]

2.2. Elastase model

All murine investigations were approved by the Leiden University Medical Center animal welfare committee and were in compliance with the Dutch government guidelines.

Eight-to-ten weeks old, male, C57BL/6 mice were obtained from Charles River, France. The aneurysms were created via porcine pancreatic elastase (PPE) infusion as previously described [17-20]. After the elastase infusion 0.05–0.1 mg/kg/12 h buprenorfine was given and the mice recovered with free access to food and water. The oral CXCR1/2 antagonist DF2156A (6 mg/kg), a generous gift from Dompé Pharma, Milan, Italy [21] was given (n = 10) daily via oral gavage in 100 μl of 0.25% carboxymethylcellulose in PBS. Treatment was started the day before the elastase infusion and the mice were sacrificed 14 days after the infusion. Control animals (n = 10) received daily oral gavage of 100 μl of 0.25% carboxymethylcellulose in PBS for 15 days.

To compare the aortic growth rates of the different groups we measured the maximum axial diameter of the aorta by means of ultrasound one day prior to elastase infusion, after one week and two weeks after infusion by means of the Vevo 770 Imaging system using RMV 704 microvisualization scan head (Visualsonics, CA).

At day 14 after the elastase infusion, the mice were sacrificed and the aorta was removed, and embedded in paraffin for later analysis. Immunohistochemical sections were deparaffinized and incubated overnight at room temperature with the primary antibody diluted in PBS -1% albumin. The sections were incubated with CD45 (BD Pharmingen, Breda, The Netherlands), MAC3 (BD Pharmingen), MMP9 (Santa Cruz Biotechnology) and MPO (Abcam). Additional sections were stained with Sirius Red for collagen and Weigert's elastin stain to visualize elastic laminae. Six slides per animal were used per staining for analysis and only moderate or strongly reactive cells were counted as positive. The slides were blindly evaluated. The mean value for positive staining cells on six slices was calculated for each animal.

2.3. Statistical analysis

All values are shown as mean (SD) and probability values of $P < 0.05$ were considered statistically significant. After performing an ANOVA test to explore the difference between human AAA and human atherosclerotic samples, an unpaired *t*-test was performed.

The Mann-Whitney *U* test was used to detect significant difference in aortic diameter and in cell count between the two groups of mice.

All analysis were performed using SPSS 23.0 (IBM, Amsterdam, The Netherlands).