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Pentraxin 3 and complement cascade activation in the failure of arteriovenous fistula

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ABSTRACT

Objective: Pentraxin-3 (PTX3) has been suggested to play a role in the development of vascular pathology. Stenosis of arteriovenous fistula (AVF) leading to its failure is the major cause of morbidity in hemodialysis patients. To date, little is known on the pathogenesis of AVF stenosis. The aim of the present study was to investigate the potential role of PTX3 in this setting.
Methods and results: A sample of venous wall was collected at the time of AVF formation in 44 patients with end stage renal disease. Ten patients developed AVF stenosis and from these patients a second portion of the venous wall was obtained during surgical revision of the AVF. Confocal laser scanning microscopy demonstrated that PTX3 immunostaining, hardly detectable in native AVF, was significantly increased in failed AVF, showing a specific co-localization with endothelial cell markers. Circulating mononuclear cells isolated at the time of AVF revision presented a significantly higher PTX3 mRNA expression than those collected during AVF creation. Interestingly, a significant deposition of C5b-9 on endothelial cells, co-localizing with PTX3, was observed in stenotic AVF.
Conclusion: The present study demonstrates for the first time a close association between PTX3 deposition and complement activation at the endothelial cell level in failed AVF and suggests a role for PTX3 in modulating innate immunity in the pathogenesis of AVF stenosis.

1. Introduction

An increasing body of evidence demonstrates that the immune system is actively involved in the development and progression of vascular pathology [1–3]. The early stages of atherosclerosis are characterized by an influx within the vessel wall of inflammatory cells probably recruited by the main soluble factors of innate immunity [4]. Pentraxins and complement are essential components of the humoral arm of the innate immune system and may play a pivotal role in vascular biology [5]. Pentraxins constitute a superfamily of evolutionary conserved proteins [5]. The recently characterized pentraxin-3 (PTX3) is the prototype of the long pentraxins, which differs from the short pentraxins for gene organization, cellular source and ligand-binding capacities. Unlike short pentraxins, PTX3 is not produced by hepatocytes but is synthesized by a variety of cell types at the site of inflammation [4,6–8]. Interestingly, PTX3 can regulate complement activation [4,9,10]. The complement system has been also implicated in the progression of atherosclerosis via different mechanisms, including the release of chemotactic factors, which drive the recruitment of inflammatory cells within the vessel wall [11]. Moreover, the local deposition of the terminal complement complex C5b-9 has been shown to induce the activation of endothelial cells [12].

Vascular access dysfunction is one of the main causes of morbidity and hospitalization in hemodialysis [13,14]. Native arteriovenous fistula (AVF) is the preferred form of vascular access because of low incidence of infections and thrombosis [14]. The main complication involving AVF is represented by the stenosis of its venous branch followed by thrombosis. Stenotic AVF is characterized by significant vascular remodeling including concentric neointima hyperplasia and may share some histological and pathogenic features with atherosclerosis [14–16]. To date, little is known on the pathogenic factors involved in AVF stenosis and there is lack of specific therapeutic interventions [14].

Aim of the present study was to investigate the potential involvement of PTX3 and complement in the stenosis and failure of AVF.

2. Material and methods

2.1. Patients

From January 1999 to December 2003, a total of 44 patients undergoing surgery for AVF creation, having given their informed

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consent, were enrolled in a prospective observational study. All patients fulfilled the following inclusion criteria: age older than 18 years and younger than 75, end-stage renal disease on conservative therapy, no previous AVF surgery, and a lower-arm AVF. They did not present any of the following exclusion criteria: diabetes, extensive vascular disease, neoplasia, inflammatory diseases, corticosteroid and/or immunosuppressive therapy. During the surgical procedure, we collected one sample of the venous wall from each patient. All patients included in the study began hemodialysis within a mean of 3 months from AVF surgery (range, 1–6 months). In this population, we observed 10 AVF failures (0.12 events/patient/year). Mean patient age was 50 years (range, 30–76 years), and mean time from the beginning of hemodialysis to AVF surgical revision was 26 months (range, 6–60 months). All patients were hypertensive with a mean of 2.4 anti-hypertensive medications (range 1-4) and receiving erythropoiesis-stimulating agents (erythropoietin alpha 3, beta 3 and darbopoietin 4). Mean serum cholesterol levels were 210 ± 35 mg/dl, with no patient on statin treatment. Mean serum C reactive protein (CRP) (high sensitivity) levels were 0.8 ± 04 ng/ml. Six out of ten patients were smokers. Underlying kidney disease leading to uremia was chronic glomerulonephritis in 3 patients, chronic interstitial nephritis in 1 patient, vascular disease in 4 patients and cystic diseases in 2 cases. We did not observe any statistically significant difference regarding the main clinical, demographic and laboratory parameters between the groups of patients with or without AVF failure (data not shown). In all patients, venous branch stenosis was confirmed by Doppler ultrasound and histologically at the time of revision (wall-lumen ratio > 1). A sample of the stenotic venous branch (not closer than 3 cm from the anastomosis) was collected from all patients during surgical revision. Failure due to an episode of hypotension was not documented for any patients. All the comparisons were performed between native and failed AVF samples obtained from the same patient. The study was approved by our Institutional Ethical Committee.

2.2. Immunohistochemical analysis

Thin (2 μm) sections of paraffin-embedded tissue were rehydrated through xylene and graded alcohol series. After antigen retrieval, the sections were blocked with a serum-free protein block solution (Dako, Glostrup, Denmark). The sections were, then, incubated with a rabbit anti-human C4d antibody (Dako) followed by a biotinylated goat anti-rabbit IgG antibody. The binding of the biotinylated antibody was detected by the G/2 System/AP kit (Dako) The sections were counterstained with Mayer haematoxylin (blue) and mounted with glycerol. Negative controls were obtained incubating serial sections with the blocking solution and then omitting the primary antibody. Section's staining was evaluated by optical light microscopy using a Leica microscope (Leica Microsystems, Wetzlar, Germany).

2.3. Tissue immunofluorescence and confocal laser scanning microscopy

PTX3/CD34 co-localization was evaluated on paraffin-embedded tissue sections, while PTX3/C5b-9 and PTX3/Von Willebrand factor co-localization was investigated on AVF frozen tissue included in OCT medium (Tissuetek, Pittsburgh, PA). The following primary antibodies were used for the confocal microscopy studies: anti-PTX3 (MNB4, Exira Life Sciences Inc., Larsen, Switzerland); anti-CD34 (Ansell, Bayport, MN); anti-C9 (aE11; Hycult biotechnology, Uren, The Netherlands); anti-Von Willebrand factor (Santa Cruz Biotechnology Inc., Santacruz, CA); anti-α smooth muscle actin (Santa Cruz Biotechnology Inc.) and anti-BDCA1 (Miltenyi Biotech, Bergish Gladbach, Germany). The mouse, rat and rabbit antibodies were detected using 488 goat anti-mouse IgG, 488 goat anti-rat IgG and 546 goat anti-rabbit IgG antibodies, respectively (Molecular Probes, Eugene, OR). Specific fluorescence was identified by confocal microscopy using the Leica TCS SP2 (Leica, Wetzlar, Germany) equipped with argon-krypton (488 nm), green-neon (543 nm) and helium-neon (633 nm) lasers. In order to stain the nuclei, samples were incubated with TO-PRO (Invitrogen-Molecular Probe, Carlsberg, CA). The slides were then mounted in Gel Mount (Biomed Corp., Foster City, CA) and sealed.

The mean fluorescence intensity (MFI) was quantified by a specific confocal laser scanner software (Leica TCS-SP2). For this purpose, two independent observers (D. Marrone and G. Dalfino) selected 8 regions of interest of the same size/biopsy section. The MFI was represented by the intensity of each pixel in the selected channel/the number of pixels included in the region of interest.

2.4. Isolation of PBMC and differentiation of monocyte-derived dendritic cells

Peripheral blood mononuclear cells (PBMC) and dendritic cells were generated as previously described [17]. After 6–7 days of differentiation in the specific medium, dendritic cells were activated by LPS [17].

2.5. RNA extraction and reverse-transcription polymerase chain reaction

Total RNA was obtained by phenol/chloroform extraction and isoamyl alcohol precipitation. Reverse transcription was carried out with 1 μg of total RNA by AMV First-Strand cDNA Synthesis kit (Invitrogen), using random hexamers, according to manufacturer's instructions. Oligonucleotide primers for PCR amplification of human PTX3 and β-actin were as follows: PTX3 forward = 5′-CAT CCA GTG AGA CCA ATG AG-3′ reverse = 5′-GTA GCC GCC AGT TCA CCA TT-3′. β-actin:forward = 5′-GGC ATC GTG ATG GAC TCC G-3′ reverse = 5′-GCT GGA AGG TGG ACA GCG A-3′. Complementary DNA amplification was performed with 1 unit of HotMaster™ Taq DNA polymerase system (Eppendorf, Hamburg, Germany) in a total volume of 25 μL. For PTX3, cDNA was diluted 10 fold. PTX3 and β-actin cDNA amplification profile involved denaturation at 94 °C for 20 s, primer annealing at 58 °C for PTX3 and at 68 °C for β-actin for 20 s and extension at 72 °C for 20 s. The number of PCR cycles used was within the linear range of reactions. PCR products and molecular markers were separated by electrophoresis in a 1.8% agarose gel in Tris borate/EDTA buffer. Expected sizes of amplified fragments were 287 bp and 612 bp for PTX3 and β-actin, respectively. Intensity of the PTX3 and β-actin bands was quantified by Image J software (NIH, Bethesda, MD). Results were expressed as PTX3 to β-actin ratios.

2.6. Statistical analysis

Data are presented as mean ± standard deviation (SD) and are compared using analysis of variance or paired Student *t*-test, as appropriate. Differences were considered statistically significant when p values were less than 0.05. Data were analyzed using GraphPad Prism (GraphPad software, San Diego, CA).

3. Results

3.1. Analysis of PTX3 signal in native and failed AVF

We first investigated the presence of PTX3 in the venous wall of native AVF. We observed a very limited PTX3 specific fluorescence in the native veins with very few PTX3+ cells (Fig. 1A and B). On the contrary, when we analyzed the stenotic AVF (Fig. 1C), we found a