Expression of C-reactive protein in myointimal hyperplasia in a porcine arteriovenous graft model

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Abstract

Background. The migration and proliferation of myofibroblasts are prominent features of myointimal hyperplasia associated with haemodialysis polytetrafluoroethylene (PTFE) grafts. Since C-reactive protein (CRP) possesses functional activities on vascular smooth muscle cells (SMCs), we examined the expression of this protein in PTFE grafts early in the course of myointimal hyperplasia development in a porcine model.

Method. Bilateral carotid-jugular PTFE loop grafts were placed in pigs. After euthanasia at 2–4 weeks, the graft-venous and graft-arterial anastomoses and the adjacent blood vessels were excised en bloc and subjected to immunohistochemical analyses and in situ hybridization for CRP. The ability of CRP to stimulate proliferation was examined in cultured porcine venous SMCs using the bromodeoxyuridine assay after incubation for 48 h.

Results. Severe myointimal hyperplasia was found at 3 weeks after graft placement at both graft-venous and graft-arterial anastomoses. Compared to normal tissues, staining for CRP was far more intense in cells in the hyperplastic lesions at both anastomoses, which also stained positive for smooth muscle α-actin. In situ hybridization showed that these cells also expressed mRNA for CRP. At 1 µg/ml, CRP increased the proliferation of cultured porcine venous SMCs by 45.9 ± 5.8%.

Conclusion. CRP was produced in venous and arterial SMCs and its expression was enhanced in the hyperplastic lesions associated with arteriovenous PTFE grafts in a porcine model. Together with the ability of CRP to promote SMC proliferation, these data suggest that CRP might play a pathogenic role in the development of myointimal hyperplasia in PTFE grafts.

Keywords: C-reactive protein; haemodialysis; myointimal hyperplasia; synthetic graft; vascular access; vascular smooth muscle cells

Introduction

Vascular accesses, in particular arteriovenous polytetrafluoroethylene (PTFE) grafts, used for chronic haemodialysis are prone to stenosis. Approximately 60% of the graft stenosis occurs around the venous anastomosis, although the arterial anastomosis is also affected. The stenosis is usually caused by myointimal hyperplasia, with the migration and proliferation of vascular myofibroblasts being the key events in its development [1]. Myofibroblasts are transformed fibroblasts that have acquired certain characteristics of smooth muscle cells (SMCs). While low shear stress, turbulent blood flow and mechanical and surgical injuries have been incriminated, the pathogenesis of neointimal hyperplasia is still incompletely understood. On the cellular level, various mediators, such as platelet-derived growth factor, basic fibroblast growth factor [1], transforming growth factor-β, endothelin-1 and tumour necrosis factor-α appear to be contributory. The role of C-reactive protein (CRP) in this context, however, has not yet been explored.

C-reactive protein (CRP) is an acute-phase reactant primarily produced in the liver, although it is also produced in other organs such as the heart, lung, kidney, spleen and adipose tissue [2,3]. Its plasma level is often used clinically to monitor inflammation and infection. In recent years, a dogma that long-term sub-clinical vascular inflammation leads to clinical cardiovascular events has evolved. Whether this dogma is accurate or not, the plasma level of CRP has become a common cardiovascular risk marker in both nondialysis chronic kidney disease [4] and end-stage renal disease (ESRD) populations.

In support of a potential role of local CRP in vascular disease, CRP expression has been demonstrated in stenotic coronary venous bypass grafts and...
atherosclerotic coronary arteries [5]. Inflammatory stimuli, such as lipopolysaccharide and interleukin-1β have been reported to enhance the production of CRP by human coronary arterial SMCs, but not human umbilical venous endothelial cells [6]. In turn, CRP activates the expression of monooyte chemoattractant peptide-1, interleukin-6 and the intracellular NK-κB, MAPK, c-Fos/cJun and AP-1 pathways in rat aortic SMCs [7]. These data provide evidence that, in addition to being a marker molecule, CRP is a functional molecule that may play a role in atherogenesis directly by activating vascular SMCs and indirectly by inducing cytokine production. The functionality of CRP leads us to speculate that this protein is also involved in the pathogenesis of another inflammatory vascular disease, namely, myointimal hyperplasia associated with haemodialysis arteriovenous grafts. In this study, we first examined the expression of CRP by the hyperplastic SMCs in a porcine model of PTFE graft stenosis.

Porcine models have been used by several laboratories for the study of PTFE graft stenosis [8]. Pigs have also been used as models for inflammation in other settings, with serum CRP levels as markers in particular, such as natural infections by porcine reproductive and respiratory syndrome virus and mycoplasma [9], turpentine-induced inflammation [10] and post-weaning multisystemic wasting syndrome [11]. Of particular relevance to the present study, CRP has been found in coronary artery plaques of pigs that are fed a high cholesterol diet [12]. Thus, porcine models are suitable not only for the study of inflammation associated with infection, but appear to be suitable for the study of low-grade inflammation associated with proliferative vascular diseases.

Methods

Porcine model of graft stenosis

The porcine model of arteriovenous graft stenosis employed in the present study has been previously described [13]. All animal procedures and care were performed in accordance with the ‘Principles of Laboratory Animal Care’ and the ‘Guidelines for the Care and Use of Laboratory Animals’ (NIH Publication No. 85-23, revised 2001). These procedures were also approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Utah and the Veterans Affairs Salt Lake City Healthcare System.

In brief, Yorkshire cross domestic swine, weighing 29.9 ± 1.4 kg were used. General anaesthesia was induced using xylazine (4 mg/kg), telazol (4 mg/kg) and ketamine (4 mg/kg) and maintained using 1.5–2.5% isoflurane. Sodium heparin (100 units/kg) was given intravenously during surgery. Under sterile conditions, expanded PTFE grafts (spiral re-enforced, 7 cm length, 6 mm internal diameter; Bard Peripheral Vascular, Inc., Tempe, AZ, USA) were placed between the common carotid artery and the ipsilateral external jugular vein bilaterally. Starting 6 days pre-operatively, aspirin EC (Pharmaceutical Formulations, Edison, NJ, USA) at 81 mg/day was administrated orally. Clopidogrel (Bristol-Myers Squibb, New York, NY, USA) 225 mg was added 1 day before surgery and continued at 75 mg/day post-operatively. Both anti-platelet agents were continued until euthanasia. Enrofloxacin (Baytril, Bayer, Pittsburgh, PA, USA) was administrated subcutaneously at 5 mg/kg/day starting on the day of surgery and continued for 3 days. Graft patency was monitored weekly using a Doppler ultrasound (TITAN transducer L38/10-5 MHz, SonoSite, Bothell, WA) while the animal was sedated. A total of four pigs with bilateral graft implantations and three untreated control pigs were sacrificed in the studies described in this report. For immunohistochemistry, one animal was sacrificed at week 2 (one graft used), two animals at week 3 (two grafts used) and one animal at week 4 (one graft used). For in situ hybridization, two animals were euthanized at week 3 (two grafts used) and one animal at week 4 (one graft used). An additional 12 pigs, totalling 16 pigs, with graft implantation were used for the determination of plasma CRP concentrations.

Immunohistochemical analysis of explanted tissues

Two to four weeks after graft placement, the animals were euthanized. Grafts were explanted en bloc along with 1–2 cm of the adjacent blood vessels, fixed in 10% buffered formalin and embedded in paraffin. Serial cross sections were subjected to histological and immunohistochemical analysis. Slides for histology were stained with haematoxylin and eosin (H&E). For immunohistochemical analysis, the slides were deparaffinized using Trilogy® (Cell Marque, Hot Springs, AR, USA) for 15 min and incubated for 40 min with a monoclonal anti-human CRP antibody (Sigma-Aldrich, St Louis, MO, USA) at 1:100 dilution or anti-human smooth muscle α-actin antibody (Dako, Carpinteria, CA, USA) at 1:100 dilution. Subsequently, the slides were incubated for 20 min with a horseradish peroxidase (HRP)-conjugated affinity-purified goat anti-mouse IgG (Envision System HRP #K1392, Dako) as the second antibody. The HRP on the slide was then developed following the manufacturer’s instructions. Murine non-immune IgG (Sigma) was used to test the nonspecific binding of IgG isotype. The external jugular veins and the common carotid arteries from untreated normal pigs were used as normal controls, while tissues from the gut, lymph node or tonsil were used as positive controls.

To examine if the CRP protein co-localized in the same cells that expressed smooth muscle α-actin, double staining was performed. CRP staining was first performed as described above, which produced a brown appearance in positive-staining cells. The slide was then washed and exposed to the anti-smooth muscle α-actin at 1:50 dilution for 30 min. An alkaline phosphatase-labelled polymer was then applied for 30 min and developed with Vulcan Fast Red for 15 min, which produced a red appearance in positive-staining cells.

In situ hybridization

The primer sequences for porcine CRP were as follows: 5'-TGAGTGGCGAGGTGTATGTC -3' (forward) bases 740–759 and 5'-GATGTGGCCCTGCTAGCTCTAA-3' (reverse) bases 962–982, (AB005545). SP6- and T7-specific
transcription factor recognition sites were incorporated into 5' ends of the forward and reverse primer sequences, respectively and used to produce biotinylated 246 bp sense and anti-sense CRP-specific riboprobes according to the manufacturer’s instructions (SP/T7 Transcription Kit, Roche).

For these studies, grafts were explanted from pigs at 3–4 weeks post-graft placement. *In situ* hybridization was performed on 10% buffered formalin-fixed, paraffin-embedded blocks of these tissues. Five-micron sections were mounted on slides, deparaffinized in three changes of xylene (10 min each) and rehydrated in a graded series of ethanol solutions (100% x 2, 95% x 2 and 70% x 1) for 1 min each. The slides were then placed in water and pretreated with Protease 1 (Ventana Medical Systems, Tucson, AZ) for 10 min at RT. After rinsing with APt wash (Ventana) for 5 min, the probe was added and the slides were heated for 10 min at 95°C. The slides were placed in a hybridization chamber and incubated for 12 h at 37°C. Following rinsing in a post-hybridization buffer for 5 min at RT, the slides were placed in an automated immunostainer (Benchmark, Ventana) and developed using the ISH IView® Blue Plus detection kit (Ventana). The slides were sequentially washed with detergent, iodine, sodium thiosulfate and water according to the manufacturer’s instructions and dehydrated in a graded series of ethanol solutions. These experiments were performed on tissues collected from three different animals with graft implantation.

*Plasma C-reactive protein levels*

Three millilitres of blood were collected in tubes containing 2% EDTA from the ear veins of 16 pigs before graft placement and weekly postoperatively. Plasma levels of CRP were measured using the enzyme immunoassay kit specifically designed for porcine CRP (Tri-Delta Diagnostics).

*Smooth muscle cell isolation, culture and proliferation assessment*

Porcine venous SMCs were harvested from three pigs and cultured as described previously [14]. Briefly, a femoral vein segment (5 cm in length) was obtained from these animals. The adventitial layer was carefully removed by dissection. Endothelial cells were detached from the segments by incubating with collagenase A (0.2 mg/ml) in RPMI for 1 h at 37°C. The SMCs were dissociated from the remaining tissue using a mixture of collagenase A (0.2 mg/ml), elastase (0.05 mg/ml) and 0.1 mg/ml of bovine serum albumin for 15 min at 37°C. The SMCs were cultured in a smooth muscle cell culture medium (Medium 231, Cascade Biologics, Portland, OR, USA) containing 20% fetal calf serum (FCS), 5 μg/ml insulin, 0.5 ng/ml human epidermal growth factor, 2 ng/ml human basic fibroblast growth factor, 100 μg/ml streptomycin, 100 U/ml penicillin G and 0.25 μg/ml amphotericin B. The identity of SMCs was confirmed by their typical morphology under light microscopy and positive staining with FITC-conjugated anti-human α-actin. Cells between passages 2 and 6 were used.

The SMCs were seeded in 96-well plates (1 x 10^3/well), cultured for 48 h and then incubated in Medium 231 supplemented with 0.5% FCS for an additional 48 h to induce cell quiescence. Incremental concentrations (0–1.0 μg/ml) of human recombinant CRP (Calbiochem, San Diego, CA, USA) in a fresh medium (Medium 231) containing 0.5% FCS without growth factors were added. After further incubation for 48 h, cell proliferation was assessed using the bromodeoxyuridine (BrdU) incorporation assay (Amersham Biosciences, Piscataway, NJ, USA) which assesses DNA synthesis.

*Statistical analysis*

Results are expressed as mean ± SEM. Paired *t*-test was used to compare plasma CRP levels with baseline values and compare BrdU incorporation in CRP-stimulated cells with controls. *P*-values <0.05 are considered statistically significant.

*Results*

**Immunohistochemistry**

There were no noticeable infections, haemorrhage or significant skin inflammation around the surgical wounds or other major problems during follow-up in the animals. As previously reported, myointimal hyperplasia developed progressively at both graft-venous and graft-arterial anastomoses starting 2 weeks after graft placement [13]. Representative histological cross-sections of the graft-venous and graft-arterial anastomoses explanted at 3 weeks stained with H&E were presented in (Figure 1).

Immunohistochemical staining was performed on graft anastomotic tissues obtained at 2–4 weeks postoperatively using an anti-human CRP as the primary antibody. There was intense and diffuse staining for CRP in the cells and extracellular matrix within the myointimal hyperplasia in both graft-venous and graft-arterial anastomoses in all three animals examined (Figure 2), with no binding of the IgG isotypic controls. Double-staining showed that CRP and α-actin co-localized in many cells (Figure 3). The CRP protein was also found in normal porcine external jugular veins and common carotid arteries, but the intensity of staining was substantially weaker. Although the staining was not quantified, the intensity was markedly different between the anastomoses and the control vessels, without any overlap.

**In situ hybridization**

*In situ* hybridization using an anti-sense probe showed prominent expression of mRNA for CRP in the myointimal hyperplastic tissues at both graft-venous and graft-arterial anastomoses (Figure 4).

*Plasma C-reactive protein levels*

Peripheral blood was collected pre-operatively (baseline) and weekly post-operatively for 3 weeks.
There was a 3.1-fold increase in plasma CRP levels at 1 week, compared with baseline (94 ± 16 μg/ml vs 30 ± 6 μg/ml; n = 16; P = 0.001) (Figure 5). The plasma level gradually decreased towards baseline in subsequent weeks, but was still significantly elevated 2 weeks after graft implantation (65 ± 11 μg/ml vs 30 ± 6 μg/ml; n = 16; P = 0.007).

Smooth muscle cell proliferation stimulated by C-reactive protein
Recombinant CRP enhanced the proliferation of cultured porcine venous SMCs. BrdU incorporation increased by 9.2 ± 7.6% and 45.9 ± 5.8% at 0.1 μg/ml and 1 μg/ml of CRP, respectively (n = 3; P = 0.005) compared with 0.5% FCS alone (Figure 6).

Discussion
We have established a porcine carotid artery-to-jugular vein PTFE graft model in which progressive myointimal hyperplasia is found at 2–4 weeks post-operatively at both graft-venous and graft-arterial anastomoses [13], similar to other published porcine models [8]. In the present study, we first observed the expression of CRP protein in the graft hyperplastic tissues (Figure 2); however, the origin of this protein in the hyperplastic vascular tissues was unclear. Theoretically, it could be derived from an exogenous source, such as the liver and deposited in the vascular tissues through the circulating blood. Indeed, peripheral plasma CRP levels were elevated during the initial 2 weeks following graft implantation (Figure 5).
Fig. 3. Double immunohistochemical staining of porcine tissue for CRP and anti-smooth muscle α-actin. The neointimal hyperplasia at the graft-venous anastomosis (panel A at 1× magnification) was stained with anti-CRP (appearing brown) and anti-smooth muscle α-actin (appearing pink). Co-localization of anti-CRP and anti-smooth muscle α-actin were observed in the adventitia (panel B at 400× magnification), neointimal hyperplasia (panel C at 400× magnification) and media (panel D at 400× magnification).

Fig. 4. In situ hybridization for CRP in myointimal hyperplastic tissues at the graft-venous and graft-arterial anastomoses. Sequential cross-sections of the hyperplastic tissues at the graft-venous anastomosis was stained with H&E (panel A) and subjected to in situ hybridization using an anti-sense (panel B) or sense (panel C) probes for CRP. The mRNA of CRP was prominently expressed in the myofibroblasts in the venous anastomotic hyperplasia (appearing blue). Similarly, sequential cross-sections of hyperplastic tissues at the graft-arterial anastomosis of the same animal was stained with H&E (panel D) and subjected to in situ hybridization using an anti-sense (panel E) or sense (panel F) probes for CRP. The mRNA of CRP was also prominently expressed in the myofibroblasts in the arterial anastomotic hyperplasia. All images are shown at 40× magnification.
The strong expression of the CRP protein in the graft hyperplastic tissues at 3 weeks (Figures 2–3) at the time that the plasma level had returned to baseline (Figure 5), however, suggests that the CRP may be produced locally instead. In situ hybridization further demonstrated the expression of mRNA for CRP in the hyperplastic tissues (Figure 4), confirming the local production of this protein. Since the majority of cells in myointimal hyperplasia is myofibroblasts that stained positive for $\alpha$-actin [1,15] and that the CRP appeared to co-localize with the $\alpha$-actin-positive cells (Figure 3), it is plausible that the vascular myofibroblasts are indeed the source of CRP in the myointimal hyperplasia associated with dialysis grafts.

The stimuli for the CRP production by SMCs within the myointimal hyperplasia are unclear. Cytokines such as IL-1$\beta$, IL-6 and TNF-$\alpha$ are well known to stimulate CRP production by hepatocytes [16] and have also recently been reported to stimulate CRP production by human coronary arterial SMCs [6]. Trauma at the anastomoses induced by the graft placement surgery, bioincompatibility of the graft material, mechanical stress induced by haemodynamic changes in the vessels and injury from repeated needle puncture could potentially stimulate the production and/or release of cytokines. Recent reports showed that the mRNA for CRP was over-expressed in atherosclerotic plaques in the human aorta [2]. In as much as atherosclerosis is an inflammatory disease [4], these reports support the notion that local CRP production in the vascular wall is enhanced by inflammation. The definitive role of inflammation vs other cellular processes in promoting CRP production by vascular SMCs would require further studies.

That CRP is a useful marker of inflammation and a predictor of cardiovascular outcomes has been well established. More recent data, however, indicate that CRP is also a functional molecule. It has been reported to increase nitric oxide production, increase NF-κB and mitogen-activated protein kinase activities in rat aortic SMCs [7], induce apoptosis in human coronary artery SMCs [17] and up-regulate angiotensin type-1 receptors resulting in increased reactive oxygen species and proliferation in human saphenous vein SMCs [18]. In the present study, we further showed that CRP stimulated the proliferation of cultured normal porcine venous SMCs in a dose-dependent manner (Figure 6). In as much as CRP is produced by myofibroblasts in myointimal hyperplasia and atherosclerotic tissues and CRP possesses functional activities in these cells, it may be considered as part of an autocrine system for these cells. CRP has also been shown to activate cell signalling and promote chemotaxis by monocytes. To the extent that monocytes infiltrating the tissues are involved in the pathogenesis of atherosclerosis [4] and neointimal hyperplasia [1], CRP may also participate in a paracrine manner in vascular disease states. A caveat of the present and other studies on the biological activities of CRP needs to be noted. The in vitro vasodilatory activities of CRP have reportedly been attributed to the sodium azide contained in the commercial preparations [19]. Whether this phenomenon also applies to the proliferative properties of CRP in the present study is unclear.

In conclusion, our present study showed the expression of CRP was constitutively expressed in normal porcine vascular SMCs and that production of this protein was greatly enhanced in both venous and arterial myofibroblasts in myointimal hyperplasia associated with haemodialysis arteriovenous grafts. CRP stimulated the proliferation of venous SMCs and may therefore participate in a paracrine system. The strong expression of CRP may contribute to the high propensity of myointimal hyperplasia development at the venous anastomosis associated with haemodialysis grafts. A number of medications, including cyclooxygenase inhibitors, platelet aggregation inhibitors, lipid lowering agents, $\beta$-adrenoceptor antagonists, antioxidants and angiotensin antagonists, have been shown to reduce serum levels of CRP [20]. Whether the
CRP-reduction effect of some of these medications has therapeutic implications warrants further studies.

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Conflict of interest statement. None declared.

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