Role of osteopontin in the development of neointimal hyperplasia in vein grafts

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INTRODUCTION

Even with the rapid development of intra-coronary stents, certain advantages of coronary artery bypass grafting (CABG) over percutaneous coronary intervention are likely to continue in the foreseeable future, particularly in diabetic patients and in those with left main and multi-vessel coronary diseases [1]. The autologous saphenous vein remains a widely used conduit for CABG. However, late vein graft failure is the Achilles’ heel of this intervention. Nearly half of patients may require reoperation 10–15 years after CABG to alleviate symptoms and treat vein graft occlusion [1, 2]. The pathophysiology of vein graft failure is complex, whereas neointimal hyperplasia, a process mediated by the proliferation and migration of vascular smooth muscle cells (VSMCs), has been recognized to be the main cause of late failure. Superimposed on neointima formation is atherogenesis, which ultimately leads to plaque rupture and graft occlusion [3].

Osteopontin (OPN) is a transformation-related phosphorylated acidic glycoprotein, which was first identified and isolated from the bone [4]. Existing as an immobilized extracellular matrix (ECM) component protein and as a soluble molecule, it has been implicated in inflammation, cell-mediated immunity, tissue remodelling, and tumour metastases [5]. A wide range of cell types including epithelia, macrophages, T cells and VSMCs express OPN in a constitutive or inducible fashion [6]. OPN functions as a cell adhesion and migration molecule that can bind to several ligands such as αvβ3 integrin, and CD44 receptors, as well as fibronectin [5, 6]. OPN has recently been proposed to play a key role in vascular injury responses and in the development of atherosclerosis [6–8]. Although VSMCs and endothelial cells synthesize OPN at very low levels, it can be over-expressed at the site of endothelial injury [9]. Such an elevated expression of OPN can promote cultured VSMCs migration and proliferation in post-injury remodelling of arterial wall in animal models [8, 10]. In particular, exogenous administration or inhibition of OPN has...
been found to stimulate or suppress the related cascades of events leading to neointima hyperplasia in human coronary arteries and saphenous veins in vitro [11]. As a cell adhesion molecule with a primitive amino acid sequence, OPN can combine with VSMCs surface integrin receptors, and facilitate cellular adhesion, proliferation and migration by further modulating the activity of matrix metalloproteinases (MMPs), which are highly responsible for degradation and accumulation of the ECM. On the other hand, blockade of OPN or its integrin receptors by neutralizing antibodies [7, 11] or OPN gene knockout [12] can effectively inhibit arterial neointimal hyperplasia and superimposed atherosclerosis.

Despite a growing body of in vivo and in vitro evidence on the importance of OPN and MMP in vascular remodelling following arterial injury, their roles in vein graft injury and arterialization remain unknown. The present study was designed to assess OPN expression in porcine vein grafts following in vivo vein–carotid artery interposition grafting, and to explore its potential relationship with MMPs activities.

MATERIALS AND METHODS

Surgical procedures

The study was approved by the ethics committee of the Chinese University of Hong Kong. In total, 16 Large White pigs weighing between 35 and 45 kg underwent bilateral saphenous vein–carotid artery interposition grafting as previously described [13–16]. The animals received humane care according to the ‘Guide for the Care and Use of Laboratory Animals’. The pigs were randomly assigned into four groups before operation, by opening the pre-sealed envelopes, while the vein grafts were to be harvested at postoperative 1, 2, 4 and 12 weeks.

The pigs were fasted for 12 h before operation. Anaesthesia was induced with ketamine (30 mg) and atropine (0.6 mg), administered intramuscularly. Following endotracheal intubation, the pigs received 4% isoflurane in 1:1 oxygen and nitrous oxide gas. Amoxycillin (Merck & Co., USA) was given intramuscularly for antimicrobial prophylaxis at a dose of 20 mg/kg prior to skin incision. The pigs also received intravenous normal saline at a rate of 6–8 ml/kg per hour throughout the operation, and were monitored with ECG and pulse oximeter. Approximately 12 cm of the saphenous vein from each pig’s right leg was dissected free of surrounding tissue and all side branches were secured with a 3-0 silk ligation. The vein was removed from the animal, rinsed in iso-osmotic sodium chloride solution (0.9 g/l) containing 2 IU/ml heparin and 50 μg/ml glyceryl trinitrate and stored in the same solution at room temperature (24°C) until needed.

Two para-sternomastoid muscle longitudinal neck incisions were made and the common carotid arteries carefully dissected from the internal jugular vein and vagus nerve within the carotid sheath. Following systemic heparinization (200 IU/kg), a segment of the common carotid artery was isolated and divided between vascular clamps, bevelling the cut ends obliquely to 45°. The saphenous vein was reversed and similarly bevelled. The end-to-end anastomoses of the vein to common carotid artery were performed using continuous 7-0 Polypropylene (Ethicon, San Angelo, TX, USA) sutures. The graft was de-aired through the suture line before the suture of the second anastomosis was tied, then the vascular clamps were removed and the graft perfused at arterial pressure. Haemostasis was checked, neck and leg wounds were closed with 2-0 polyglycolic acid sutures (Davis & Geck, Hampshire, UK) and inhalational anaesthetic agents were discontinued. Pigs were extubated and, when in a satisfactory condition, returned to their pens and fed a normal chow diet.

After the required postoperative period, each vein graft together with 1 cm segment of the proximal and distal carotid artery was removed under general anaesthesia. The harvested vein graft was first rinsed with normal saline and then divided into two segments with equal length. One segment was snap frozen and stored in −70°C, while the other part was fixed in 10% buffered formalin.

Immunohistochemistry

Graft tissue was fixed in buffered formalin and paraffinized for immunostaining. Three antibodies included rabbit polyclonal anti-OPN (Abcam), mouse monoclonal anti-PCNA (Dako Ltd, High Wycombe, Bucks, UK) were used. Briefly, 5 μm section was deparaffinized and rehydrated, following antigen retrieval with 100°C citrate buffer and removal of endogenous peroxidase with 3% hydrogen peroxide. After that, the section was first incubated with appropriate dilutions of OPN (1:100) and PCNA (1:400), then incubated in corresponding horseradish peroxidase-conjugated secondary antibodies. The controls were performed by omitting the primary antibody. The expression was visualized in brown colour with the use of diaminobenzidine as a substrate, following the counterstain of nuclei with haematoxylin. Images were examined under light microscope (Ziess SPOT).

Western blot

Protein extract was obtained by homogenization of frozen graft tissue in radioimmunoprecipitation buffer. Protein concentration was determined by Bio-Rad protein assay. Under reducing condition, 25 μg of protein from each sample was loaded into 10% sodium dodecyl sulphate–polyacrylamide gel (SDS–PAGE) for the electrophoresis. The separated proteins were then blotted onto Hybond nitrocellulose membrane (Amersham). After that, the membrane was blocked for 1 h with 5% non-fat milk in Tris-buffered saline (pH 7.4) with 0.1% Tween 20 and was then incubated with either rabbit polyclonal anti-OPN (1:1000; Abcam) or sheep polyclonal anti-MMP-1 (1:1000; Abcam) at 4°C overnight. The blot was further incubated with HRP-conjugated secondary antibodies, following signal development using enhanced chemiluminescence detection. Band intensities were normalized with actin (1:1000; Santa Cruz) expression and were quantified using ImageJ software.

Gelatin zymography

MMP-2 and -9 activities were determined by MMP zymography as described elsewhere. Briefly, 15 μg of protein extract was loaded into 10% SDS–PAGE gel electrophoresis with 0.2% gelatin under non-reducing conditions. After that, the gel was washed with 2.5% Triton-X 100 for 1 h and was then incubated in developing buffer at 37°C overnight. Then, the gel was stained with 0.25% Coomassie blue R-250 for 30 min, following destaining procedure with destaining solution (10 acetic acid and 50% methanol) until sharp clear bands appeared against blue
background. The gel image was then scanned with an Epson Scanner 4990 and analysed with ImageJ software.

**Cell proliferation**

Proliferating cell nuclear antigen (PCNA) was detected in the sections by immunocytochemistry using a primary monoclonal antibody (PC10, Dako Ltd, High Wycombe, Bucks, UK) at a 1/100 dilution. This was followed by a 1/50 dilution of biotinylated anti-rabbit IgG (Dako) and avidin-biotin-peroxidase conjugate (Dako) according to the manufacturer’s instructions. Sections were counterstained with Harris’ haematoxylin. Total number of cells and number of strongly positive nuclei were counted in both intima and media in five microscopic fields per section using a ×40 objective.

**Statistical analysis**

Values are presented as mean ± standard error of the mean, or medians and inter-quartile ranges. All data were stored and analysed using a standard computer statistical software program (Statistical Package for the Social Sciences 12.0; SPSS Inc., Chicago, IL, USA). The non-parametric analyses were chosen since the result of Bartlett’s test indicated non-equal variances. In particular, the Kruskal–Wallis test was applied for comparing the time-dependent expressions of OPN and MMPs in grafted veins with those in the control group. The Mann–Whitney U-test with the Bonferroni adjustment (0.05/4) was used to measure the inter-group differences between each of the post-grafting group and the control group. The Spearman rank correlation coefficient was then used for determining the relationship between OPN expression and either the expression of MMPs, or the number of PCNA-positive cells. Statistical significance was inferred at two-sided P-values <0.05.

**RESULTS**

No major complications occurred during and after the operations. In the 12-week group, one pig died of pneumonia 2 months after surgery. Eventually, 30 patent grafts were removed after 1, 2, 4 and 12 weeks, respectively. Meanwhile, eight segments of saphenous vein taken immediately after harvesting (prior to the implantation) were used as the baseline controls. In total, there were eight grafts in all groups except in the 12-week group (n = 6).

**Expression and distribution of osteopontin protein in the venous wall**

The expression of OPN at different time points after implantation was determined by immunostaining and western blot assay. As shown in Fig. 1, the OPN expression peaked at the first week after surgery and gradually declined from the second postoperative week. Nevertheless, the expression of OPN remained significantly higher than its baseline level at all time points. It is noteworthy that the distribution of OPN protein in the venous wall presented in an outward fashion during the initial 2 weeks after implantation, from mainly in the intima in the first week (Fig. 2B) moved to the media over the second week (Fig. 2C).

**Evaluation of matrix metalloproteinases activities**

As demonstrated in the gelatin zymogram and scale bar (Fig. 3), the expression of MMP-2 and -9 followed a similar trend over the study period. They both reached peak levels by the first week after implantation, and then gradually declined from the second week postoperatively. Compared with the controls (after the Bonferroni adjustment), pro-MMP-2 expression increased significantly at all time points, while the significant up-regulation of pro-MMP9 and active-MMP-2 expression after surgery were found at 2 and 4 weeks, respectively (Fig. 3B–D). Correlations between the expression of OPN protein and pro-MMP-2, active-MMP-2 as well as pro-MMP-9 were significant (P = 0.003, 0.016 and 0.038, respectively).

**Vascular smooth muscle cell proliferation**

Proliferating VSMCs were identified by immunohistochemistry using anti-PCNA antibodies at different time points postoperatively. Compared with the control group (Fig. 4A), the number of PCNA-positive cells in the medial layer of vein graft increased significantly at the first week postoperatively (Fig. 4B), and then decreased back to the baseline level after 12 weeks (Fig. 4C–E). Such a time-dependent trend also correlated with the expression of OPN protein (r = 0.61, P = 0.005).

**DISCUSSION**

Our data showed significant OPN expression in the process of remodelling in vein grafts. We also observed a time-dependent redistribution of OPN, likely related to the essential cellular responses in the venous wall. Meanwhile, increased MMPs activities (known to be responsible for VSMCs migration and ECM...
reorganization) correlated well with the fluctuating expression of OPN during the entire study period. These findings suggest an important role of OPN in the induction and development of neointimal hyperplasia in vein grafts.

The remodelling process of vein graft starts from injury to its endothelium [3]. At the site of injury, exposed subendothelial matrix can rapidly attract the adhesion and aggregation of platelets and leucocytes. Within days after implantation, infiltrated leucocytes can release inflammatory cytokines including the multi-functional phosphoprotein OPN [6]. In fact, O’Brien et al. [17] found that infiltrating macrophages were a major source of OPN in rat and human post-injury arteries. We observed a marked accumulation of OPN confined to the intima over the first postoperative week. An early surge of apoptosis of medial VSMCs, as proposed by previous observations [3, 13], might contribute to a less evident accumulation of OPN in the media.

Figure 2: Immunostaining of OPN on vein grafts at different time points after implantation: (A) before operation, (B) 1 week, (C) 2 weeks, (D) 1 month and (E) 3 months. The scale bar in B represents 1 mm and is applicable to B–D. In A, diaminobenzidine as substrate and nuclei counterstain with haematoxylin. Neointima (NI), media (M), and adventitia (A). (microscopic fields ×40 objective).

Figure 3: (A) Gelatin zymogram for pro-MMP-9, pro-MMP-2 and active-MMP-2 at different time points after surgery. (B–D) Expression changes of pro-MMP-9, pro-MMP-2 and active-MMP-2 at different time points. Data are presented as medians and inter-quartile ranges. NS: not significant.
the following 1–2 weeks, medial VSMCs may transform into a
synthetic, migratory and proliferative status, which may be trig-
gerred by increased mechanical stretch and inflammatory cyto-
kines. These synthetic and proliferative VSMCs could be
responsible for the observed accumulation of OPN in the media
over the second postoperative week in our study. Subsequent
migration of VSMCs from the media into the intima was also
evident, in association with the deposition of large amounts of
ECM and collagen tissue within the first 3 months. Although no
obvious difference in distribution of accumulated OPN was
observed at 4 weeks in our study, we noticed significant OPN
accumulation in the formed neointima which was considered as
the ‘soil’ for atherosclerosis after vein graft implantation. In some
previous studies, high levels of OPN mRNA and protein were
detected in human coronary atherosclerotic specimens [17, 18].
Thus, our findings of local increases in accumulation of OPN
may contribute to the enhanced VSMCs proliferation and
migration.

Despite the unique biological responses inherent to venous
and arterial vessel, they share common elements involved in the
formation of neointimal hyperplasia and superimposed ather-
sclerosis [3, 13]. In particular, OPN has recently been implicated
in vascular remodelling [6–10]. As demonstrated in rat and
human aorta and carotid arteries, high levels of OPN mRNA and
protein were detectable during neointima formation [11, 12, 17,
18]. Moreover, many studies suggested that OPN can affect the
biology of in vitro cultured arterial VSMCs following integrin
engagement [9, 10]. Jalvy et al. [19] showed that autocrine expres-
sion of OPN produced by VSMC activate itself during migration.
OPN could also stimulate VSMC proliferation and migration
through more complex pathways [8, 11]. Although these findings
are based on arteries, we could speculate that OPN plays an
important role in the pathological processes associated with vein
grafts neointima formation. Our in vivo data demonstrated a
significant correlation between up-regulated expression of OPN
and the amount of PCNA-positive VSMC nuclei within
weeks after grafting. This finding supports the presence of
OPN-mediated VSMC proliferation in the vein grafts. However,
the complicated interactions between OPN and VSMCs
responses in venous wall require further investigation.

MMP family is a group of ECM-degrading enzymes that play a
crucial role in VSMC’s migration and proliferation. Their proteo-
lytic activity is inhibited by tissue inhibitors of metalloproteinases
(TIMP), especially TIMP-3 [20]. The MMP-2 and -9 are central to
the vascular remodelling processes and vein graft adaptation to
arterial environment [21]. The MMP-9, produced by endothelial
cells and surface adherent monocytes, appears to have a unique
role facilitating inflammatory cell transmigration into venous wall
in both in vivo and in vitro studies [22]. In the current study, we
found the MMP-9 expression were significantly increased in the
first 2 weeks but declined thereafter. Meanwhile, the MMP-2
was found to be critical for VSMC migration into the neointima
[22, 23]. During the remodelling process, the pro-MMP-2 is
transformed into its active form, the active-MMP-2. Our study
found a significant increase in pro-MMP-2 and active-MMP-2
during the first postoperative month, which supported this pro-

tease’s specific role in VSMC migration. At the end of the third
month, only pro-MMP-2 expression was found to be significant-
ly elevated. It is possible that less pro-MMP-2 was activated for
matrix degradation and, consequently, the MMP-2 activity
decreased.

OPN is a substrate for several extracellular proteases and can
be cleared by MMPs and other proteases. The relationship
between MMPs and OPN has been studied in the process of
post-injury remodelling in arterial vessels, which showed over-
lapping expression patterns of OPN and MMP-2 and -9 [23]. Our
study identified a similarly related expression pattern among
these mediators in vein grafts. Using neutralizing antibodies
against OPN or its αvβ3 integrin receptor, Leali et al. [9] were
able to block MMP-2 activity and VSMC proliferation, suggesting
the close interactions between OPN and MMPs.

Our observation was based on a large animal model with
modest subject numbers. Nevertheless, this is the first in vivo ex-
periment investigating the time-dependent expression of OPN

Figure 4: Immunostaining of PCNA on vein grafts at different time points after surgery: (A) before operation, (B) 1 week, (C) 2 weeks, (D) 1 month and (E) 3
months. Representative PCNA-positive staining cells (brown, examples arrowed). The scale bar represents 1 mm and is applicable to all images. (counterstain
Harris’ haematoxylin, microscopic fields ×40 objective)
protein in the process of arterial pressure-stimulated adaptation of vein grafts, and its relation to MMPs in vascular remodelling following vein graft implantation. While both surgical [24] and pharmaceutical [25] treatments are continually being refined, future studies on the mechanisms of OPN and its interaction with MMPs during venous arterization may open new horizons in therapeutic interventions targeting on neointima formation and atherosclerosis in vein grafts.

In short, the expression of OPN was up-regulated significantly in our in vivo porcine model of saphenous vein-carotid artery interposition grafting. Moreover, OPN’s time-dependent accumulation correlated well with the expression of MMP-2 and -9, which are important mediators in promoting neointimal hyperplasia. Our findings demonstrated that OPN may play a role in the development of neointimal hyperplasia, likely as an active regulator of VSMCs proliferation and migration. Whether OPN inhibition represents a novel therapeutic intervention in preventing vein graft failure deserves further investigation.

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[6] Giachelli CM, Bae N, Almeida M, Denhardt DT, Alpers CE, Schwartz SM. Osteopontin is up-regulated significantly in our in vivo porcine model of saphenous vein-carotid artery interposition grafting. Moreover, OPN’s time-dependent accumulation correlated well with the expression of MMP-2 and -9, which are important mediators in promoting neointimal hyperplasia. Our findings demonstrated that OPN may play a role in the development of neointimal hyperplasia, likely as an active regulator of VSMCs proliferation and migration. Whether OPN inhibition represents a novel therapeutic intervention in preventing vein graft failure deserves further investigation.


