African Journal of Pharmacy and Pharmacology Vol. 6(29), pp. 2235-2240, 8 August, 2012
Available online at http://www.academicjournals.org/AJPP
DOI: 10.5897/AJPP12.580
ISSN 1996-0816 ©2012 Academic Journals

Full Length Research Paper

Significance of the distribution of platelet-derived growth factor B chain in arteriosclerosis plaque in type-2 diabetic patients with arteriosclerosis obliterans of lower extremity

Xu Li, Long-Hua Fan*, Jian-Jun Liu and De-Chun Xu

Department of Vascular Surgery, Qingpu Branch of Zhongshan Hospital, Fudan University, Shanghai 201700, China.

Accepted 13 July, 2012

This study aims to explore the expression of platelet-derived growth factor (PDGF) in arteriosclerosis plaque on type 2 diabetes mellitus (DM) with arteriosclerosis obliterans (ASO) of the lower extremity. A total of 24 samples from 12 patients with lower-extremity ASO who underwent surgical treatment were collected in this study. Among these samples were 12 iliofemoral arterial plaques and 12 popliteal or distal arterial plaques. Immunohistochemistry staining was performed on the plaque intima to determine the expression level of platelet-derived growth factor receptors α and β. Reverse-transcription polymerase chain reaction was carried out to detect the mRNA level of the PDGF A and B chains. PDGFR-α and PDGFR-β were excessively expressed in lower-extremity arteriosclerosis plaques. The mean PDGF B chain mRNA level in the popliteal arterial plaques of ASO patients with type-2 DM was increased when compared with that in the iliofemoral arterial plaques. The PDGF B chain mRNA level was higher in the popliteal arterial plaques in type-2 DM with ASO than in iliofemoral artery plaques. This finding confirmed that the PDGF B chain has a key function in arteriosclerosis development.

Key words: Arteriosclerosis obliterans, platelet-derived growth factor, type-2 diabetes.

INTRODUCTION

Arteriosclerosis obliterans (ASO) refers to the arteriostenosis and occlusion caused by the accumulation of atherosclerotic material, which results in thrombosis. Type-2 diabetes mellitus (DM) patients are at risk for ASO pathogenesis 11 times higher than the general population. Type-2 DM patients also show more severe pathological symptoms, poorer prognoses, higher amputation rates, and higher death rates (Mutirangura et al., 2008).

In the ASO pathogenic progression, a large amount of platelet-derived growth factor (PDGF) can be secreted via autocrine and paracrine secretions by damaged epithelial cells, activated platelets, mononuclear macrophages, and the phenotypically transformed smooth muscle cells. Once PDGF binds to its receptor, the proliferation of mononuclear cells is enhanced, cell attachment to the intima is promoted, and cells migrate toward the subintima. The growth and proliferation of epithelial cells, fibroblasts, and vascular smooth muscle cells (VSMCs) are also PDGF dependent. The formation of foam cells from epithelial cells, fibroblasts, and VSMCs due to excessive lipid endocytosis has a key function in the occurrence and progression of arteriosclerotic plaques (Edelberg et al., 2002). The high serum glucose levels in diabetic patients may induce glycosylation of hemoglobin, causing hypoxia and lipid hypermetabolism which may further damage vascular endothelial cells (van den Oever et al., 2010; Bakker et al., 2009). Platelets form a major defense system that can be triggered by subepithelial components such as collagen fiber and von Willebrand factor. Activated platelets induce vasculitis, arteriosclerosis, and thrombus via P-selectin glycoprotein

*Corresponding author. E-mail: longhuafan@yeah.net. Tel: 86-21-69719190.

-1-mediated neutrophil and monocyte adhesion (Furie and Furie, 2004). Activated platelets also enhance the formation and progression of atherosclerotic plaques by facilitating the release of mitogen as well as chemokines such as PDGF and TGF-β (Karvinen et al., 2009; Brown et al., 2010; Doran et al., 2008). It has been demonstrated that celastrol inhibits atherogenesis in celastrol-treated apoE-mice fed an atherogenic diet by inhibiting inflammation in the arterial wall without improving the lipid profile (Cheng et al., 2011), and avocado fruit pulp (AFP) possesses hypocholesterolemic and antioxidant properties due to its phytoconstituents contents and substantiates its use in folkloric practices to control dyslipidemia (Mohammed and Al-Dosari, 2011). In addition, Mahbubeh et al. (2011) demonstrated that verjuice could reduce some atherosclerotic risk factors in long term treatment.

DM patients with lower extremity ASO typically contract vascular occlusions at remote ends of the cardiovascular circulation, which may further aggravate the ischemia symptom. Reports on the pathogenic correlation of the expression level of PDGF and its receptors with the pathogenic location and severity are limited, which necessitates the present study and related ones. In this study, we explored the expression of PDGF in arteriosclerosis plaque on type-2 DM with ASO of the lower extremity.

MATERIALS AND METHODS

Samples

A total of 24 arteriosclerosis samples from 12 patients (8 males and 4 females) were acquired in our hospital from January, 2007 to December, 2010. The average age of the patients was 76 years (range = 68 to 86 years). The patients suffered from many concomitant diseases such as high blood pressure, coronary heart disease, carotid artery stenosis, and cerebral lacunar infarction. All patients received clopidogrel (75 mg/day) and novolin therapy (Table 1). Patients (9) underwent femoral-popliteal artery bypass surgery and 3 patients underwent above-knee amputation. Among the samples were 12 iliofemoral arterial plaques and 12 popliteal or distal arterial plaques. All patients gave written informed consent, and the local ethics committee approved the study.

Assay methods

All operations were carried out under general anesthesia or continuous epidural anesthesia. During the femoral-popliteal artery bypass surgery (vascular prosthesis or great saphenous vein), proximal and distal samples were taken from the endarterectomy. Samples from the above-knee amputation patients were acquired by collecting 2 cm of the proximal superficial femoral artery and the distal popliteal artery. All samples were immediately split after acquisition. One part was stored at 4% formalin solution; the other part was mounted in a cryovial for liquid nitrogen freezing, and subsequently stored in a -70°C freezer.

Immunohistochemistry

Formalin samples were washed and desiccated before wrapping in paraffin wax. After hematoxylin and eosin (HE) staining, microscopic morphological recognition of VSMCs was carried out by identifying the nuclei as blue, cytoplasm as magenta or pink, and collagen as pink.

Expressed PDGF receptors α and β were immunostained by the following procedures. Samples were dewaxed and rehydrated before washing three times in phosphate-buffered saline (PBS, pH 7.4) for 3 min each time. Antigen was retrieved by microwave incubation at 90°C in 10 M citric acid buffer for 20 min. After washing, the samples with distilled water, endogenous peroxidase, was quenched by adding one drop of 0.3% hydrogen peroxide and was incubated at 37°C for 30 min. The samples were again washed with PBS and incubated at room temperature with 10% horse antiserum for 30 min. After incubating the samples with primary antibody (1:50 dilution of anti-PDGFR-α, 1:25 dilution of anti-PDGFR-β, rabbit anti-human monoclonal antibody; Santa Cruz BIO, USA) at room temperature for 60 min and then at 4°C overnight, they were washed again with PBS three times. The slides were then incubated with 50 µl of MaxVision^{TM} (KIT-5004 HRP-polymer anti-rabbit IHC Kit, Maixin BIO, Fujian, China) at room temperature for 45 min (9) and were washed with PBS three times. Microscopic examination was performed after adding 3,3'-diaminobenzidine (DAB) reagent (Biomiga, USA). The slides were HE stained, washed with PBS, desiccated with ethanol, and then sealed with neutral gum. The stained images were verified by examining the staining colors, that is, blue for VSMC nuclei, light pink for cytoplasms, and brown granular particle deposition for PDGFR.

Reverse transcription-polymerase chain reaction (RT-PCR)

Ribonucleic acid (RNA) was extracted by the standard Trizol method (Trizol Reagent, TakaRa) from the samples stored at -70°C. RNA was quantified before being reverse transcribed into cDNA. The reaction system (20 µl total volume) contained the following: 0.1 M dithiothreitol (DTT; Invitrogen, USA), 2 µl; 40 U/µl RNasin (Promega; USA), 0.5 µl; 10 mM 2'-deoxynucleoside 5'-triphosphate (dNTP; TakaRa, Japan), 1.0 µl; Random Primer (TakaRa, Japan), 1.0 µl; 5 × first-strand buffer, 4.0 µl; 5 U/µl DnaseI (TakaRa; Japan), 1.0 µl; and RNA, 10.5 µl. The reaction was carried out at 37°C for 30 min and 75°C for 10 min. The reaction system for reverse transcription contained the following: 0.5 µl of 40 U/µl RNasin and 0.5 µl of 200 U/µl reverse transcriptase SSII (Invitrogen, USA). The reaction was carried out at 25°C for 10 min, 42°C for 1 h, 52°C for 15 min, and 70°C for 15 min. The reaction was then stored at 4°C.

cDNA (1.0 µl) was collected from each sample for fluorescent qPCR assay. The reaction system contained the following: 5 × R-PCR buffer (TakaRa, Japan), 5.0 µl; 250 mM Mg^{2+}, 0.3 µl; 10 mM dNTP (TakaRa; Japan), 0.75 µl; 10 µM forward primer (CASarray), 0.5 µl; 10 µM reverse primer (CASarray), 0.5µl; 25 × SYBR Green I (Bio-Rad; USA), 1.0 µl; 10^{-3} calibration (Bio-Rad), 1.0 µl; 5 U/µl HS-ExTaq (TakaRa; Japan), 0.25 µl; ddH_2O, 14.7 µl; and cDNA, 1.0 µl. The reaction program was as follows: 95°C for 90 s, 95°C for 5 s + 58°C for 30 s (40 cycles), 95°C for 1 min, 58°C for 1 min, and 58°C for 10 s (+0.5°C cycle^{-1} × 68 cycles). The reaction was stored at 4°C. The primer sequences were as follows: PDGF A chain forward, 5'-CTACGGTGACCTGGTGGACT-3'; PDGF A chain reverse, 5'-ACTCGTCCTTGCTCATGTCC-3' (product size: 188); PDGF B chain forward, 5'-TACGGCAATGGCTTT ATCAC-3'; PDGF B chain reverse, 5'-CCCTCCTGCAACTTCTCAAT-3' (product size: 209 bp); and GAPDH forward, 5'-AAGGTCGGAGTCAACGGATT-3'; GAPDH reverse, 5'-CTGGAAGAT GGTGATGGGATT-3' (product size: 222 bp).

Statistics analysis

The mRNA expression level was summarized into target mRNA

Table 1. Patients' data.

Patient	Sex	Age	Preoperative fasting plasma glucose levels (mmol/L)	Symptom	Operation	Medications
1	M	86	7.1	Rest pain (R)	Bypass	Clopidogrel and Novolin50R
2	M	73	11.0	Foot (L) gangrene	Amputation	Clopidogrel and Novolin30R
3	M	77	8.5	Intermittent claudication (R)	Bypass	Clopidogrel and Novolin30R
4	M	73	8.4	Rest pain (L)	Bypass	Clopidogrel and NovolinR
5	M	82	9.8	Foot (R) gangrene	Amputation	Clopidogrel and Novolin50R
6	M	71	10.1	Rest pain (L)	Bypass	Clopidogrel and NovolinR
7	M	70	6.9	Intermittent claudication (L)	Bypass	Clopidogrel and NovolinR+N
8	M	68	8.6	Rest pain (R)	Bypass	Clopidogrel and Novolin30R
9	F	76	9.2	Intermittent claudication (L)	Bypass	Clopidogrel and Novolin50R
10	F	79	7.1	Rest pain (R)	Bypass	Clopidogrel and Novolin30R
11	F	76	13.5	Foot (R) gangrene	Amputation	Clopidogrel and Novolin30R
12	F	81	7.9	Rest pain (R)	Bypass	Clopidogrel and Novolin30R

copies against every 106 copies of GAPDH. Statistical Package for Social Sciences (SPSS) 10.0 software was used for the related analysis.

RESULTS

HE staining

In patients with type-2 DM plus ASO, HE staining showed significant VSMC hyperplasia and disordered cell alignment with some inflammatory cell penetration, as well as considerable lipid penetration around iliofemoral vascular plaques (Figure 1a). Among the popliteal arteriosclerotic samples, HE staining showed disordered VSMC alignment, with similar lipid penetration as the iliofemoral samples (Figure 1b).

Expression levels of PDGFR-α and -β

Immunostaining revealed that PDGFR-α accumulated in the shuttle-shaped stripe. In patients with type-2 DM plus ASO patients, PDGFR-α was highly expressed in plaques at the iliofemoral section (Figure 1c), with increased expression in plaques at the popliteal section (Figure 1d). PDGFR-β demonstrated granular accumulation in plaques. The expression in plaques at the iliofemoral and popliteal sections was significantly increased (Figure 1e and f).

PDGF A and B chains mRNA expression

The average PDGF A chain mRNA level in type-2 diabetes combined with ASO in iliofemoral arteriosclerosis was 26 408 ± 3447 copies per 106 glyceraldehyde-3-phosphate dehydrogenase (GAPDH) copies. The corresponding average in popliteal obliterans patients was 27 725 ± 5127 copies per 106 GAPDH copies. The difference in the two averages is statistically insignificant ($P > 0.05$). The average PDGF B chain expression level in iliofemoral arteriosclerosis samples was 102 633 ± 65267 per 106 GAPDH copies, which is far lower than that in the popliteal samples at 135 541 ± 96196 per 106 GAPDH copies. The difference is significant ($P < 0.05$) (Table 2).

DISCUSSION

PDGF is a group of mitogen and chemokine in serum with a molecular weight range of 28 to 35 kDa. The currently identified peptides in the PDGF family include four, EST ID PDGF-A, -B, -C, and -D. Functional PDGF molecules are normally homodimers or heterodimers constituted by any two of the four PDGF peptide types. Hyperplasia VSMC has been shown to express PDGF A chain and PDGF receptor gene, as well as secrete bioactive PDGF-like molecules. Thus, the expression of PDGF aggravates arteriosclerosis and further leads to obliterans (Doran et al., 2008).

PDGF receptor is a single-chain cross-membrane tyrosine kinase receptor with a molecular weight range of 170 to 180 kDa. The receptor includes two types of subunits, namely, α and β, and is functional as either a monomer or an instable dimer. PDGFR-α binds PDGF-A, -B, and -C, whereas PDGFR-β binds PDGF-B and -D. PDGF homodimer CC and DD can both bind PDGF receptor heterodimer αβ. PDGFR-α has an important function in the regulation of neural ridge cell development and metamere development in the embryonic period (Tallquist and Sodano, 2003), whereas PDGFR-β is involved in vascular wall development (Winkler et al., 2010). The expression of both PDGFR-α and -β has been

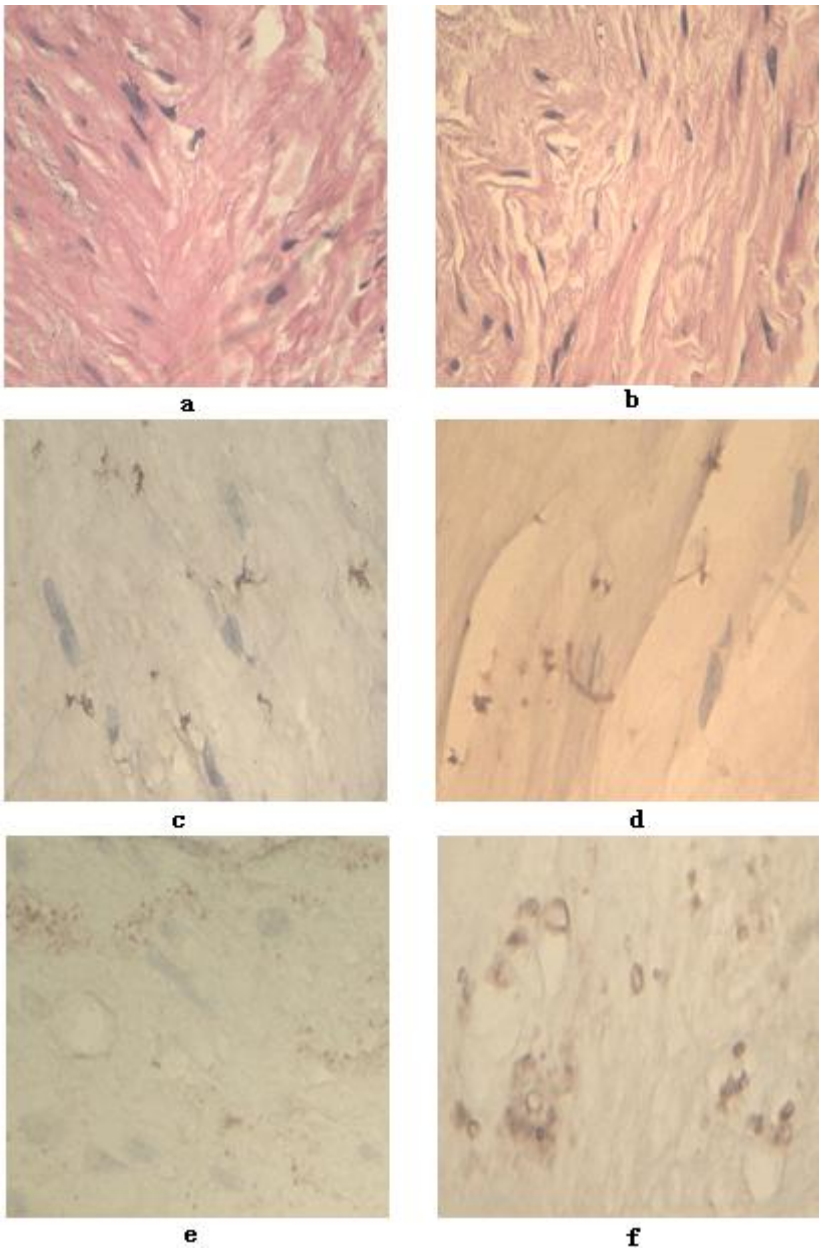

Figure 1. Hematoxylin and eosin staining. Significant VSMC hyperplasia and disordered cell alignment with some inflammatory cell penetration as well as considerable lipid penetration around iliofemoral vascular plagues (a). Popliteal arteriosclerotic samples disordered VSMC alignment, with similar lipid penetration as iliofemoral samples (b). PDGFR-α was accumulated in shuttle shaped stripe. Among type-2 diabetes induced ASO patients, PDGFR-α was intensively expressed in plagues at iliofemoral section (c), with increased expression in plagues at popliteal section (d). PDGFR-β demonstrated granular accumulation in plagues at femoral artery, with significant expression increase in plagues at iliac and popliteal section (e, f).

shown to increase in the vessels of atherosclerosis patients, and the mechanical stimulation of smooth muscle cells induces increased expression of receptors to PDGFR-α (Zhang and Khachigian, 2010).

Clinical studies revealed that arteriosclerosis is one of the major causes of amputation due to its rapid pathogenic progression, accompanied with iliofemoral obsterans, popliteal obsterans, tissue ischemia, ulcer, and necrosis. Related investigations on arteriosclerosis pathogenic mechanism, particularly, distal

Table 2. PDGF A chain and B chain mRNA expression level comparison between type-2 diabetes iliofemoral arteriosclerosis samples and popliteal artery samples.

S/N	PDGF A chain		PDGF B chain	
	Iliofemoral	Popliteal	Iliofemoral	Popliteal
1	1.96×10^4	2.35×10^4	2.65×10^4	3.29×10^4
2	3.09×10^4	3.38×10^4	2.10×10^5	2.86×10^5
3	2.67×10^4	2.24×10^4	8.01×10^4	1.22×10^5
4	2.65×10^4	3.02×10^4	6.07×10^4	6.48×10^4
5	2.67×10^4	2.15×10^4	8.44×10^4	9.14×10^4
6	2.44×10^4	2.63×10^4	7.33×10^4	9.25×10^4
7	2.15×10^4	2.71×10^4	8.12×10^4	1.05×10^5
8	3.02×10^4	3.35×10^4	1.96×10^5	2.97×10^5
9	2.97×10^4	3.58×10^4	2.16×10^5	2.89×10^5
10	2.73×10^4	2.59×10^4	6.75×10^4	7.84×10^4
11	2.45×10^4	2.12×10^4	5.37×10^4	6.88×10^4
12	2.89×10^4	3.15×10^4	8.22×10^4	9.87×10^4
M	26408 ± 3447	27725 ± 5127	102633 ± 65267	135541 ± 96196
P	P > 0.05		P < 0.05	

arteriosclerosis, may improve the rate of limb salvation. The tissue-specific pathogenic mechanism of diabetic arteriosclerosis is not yet clear. Genetics, immunological specificity, environment, and living pattern may all be effective pathogenic elements, and the related research is also sociologically importance (Boulton, 2004; Vuorisalo et al., 2009; Edmonds, 2006).

The key function of PDGF in general arteriosclerosis pathogenesis is the major consideration of the present study. The specific functionality of PDGF in lower-extremity ASO featured by distal-end vascular obliterans and severe ischemia is under debate.

Conclusion

Conclusively, the results showed that the mean PDGF B chain mRNA level in the popliteal arterial plaques of ASO patients with type-2 DM was increased when compared with that in the iliofemoral arterial plaques. The PDGF B chain mRNA level was higher in the popliteal arterial plaques in type-2 DM with ASO than in iliofemoral artery plaques. The PDGF B chain mRNA level was higher in the popliteal arterial plaques in type-2 DM with ASO than in iliofemoral artery plaques. The increased PDGF B chain expression shows that the PDGF B chain may be closely involved in the pathogenesis of distal end obstruction.

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