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Platelet Derived Growth Factor-A mRNA Levels in Diabetic and Nondiabetic Subjects at Risk of Coronary Heart Disease

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Abstract: Coronary heart disease (CHD) is the number one cause of death in developed countries. We investigated the quantity of platelet derived growth factor-A mRNA (PDGF-A mRNA) among four different groups of diabetic and nondiabetic Saudi subjects (n = 40) at risk of CHD. Total RNA was derived from peripheral blood mononuclear cells (PBMN) and PDGF-A mRNA reverse transcribed into cDNA in the presence of internal standard (cRNA) and amplified by competitive reverse transcriptase polymerase chain reaction (cRT-PCR). The gel was silver stained after polyacrylamide gel electrophoresis (PAGE) and PDGF-A mRNA quantitated by scanning densitometry. We demonstrate a significantly high expression of PDGF-A mRNA in hyperlipidaemic and diabetic subjects ($41 \pm 4 \times 10^4$ copies/µg of RNA) compared to normal healthy individuals ($29.3 \pm 5 \times 10^4$ copies/µg of RNA). These findings suggest an important role of PDGF-A mRNA in progression of CHD among diabetics.

Key words: PDGF-A, mRNA quantitation, cRT-PCR, CHD, Diabetes mellitus, Atherosclerosis.

INTRODUCTION

In Western population, coronary heart disease (CHD) is a leading cause of death[1] and its incidence is increasing in the Arab world including Kingdom of Saudi Arabia. CHD results from a disease process called atherosclerosis and several associated factors have been reported. The *in vivo* studies support the idea that platelet-derived growth factor (PDGF) may be involved in atherosclerosis[2-4]. Some previous studies have shown a high expression of PDGF-A gene in individuals with atherosclerosis and a two fold increase in plasma PDGF was found in hypercholesterolaemic patients with proven coronary atherosclerosis[5,6]. The biological properties of PDGF include stimulation of DNA synthesis, cell migration, endocytosis and secretion of matrix components[7]. PDGF from human platelets is a cationic glycoprotein of $Mr \approx 30{,}000$[8,9]. The purified PDGF is a dimer of two polypeptides, designated A and B, the products of related but separate genes. cDNA encoding the A chain of PDGF was first isolated from a malignant glioma cell line. The A-chain is encoded by a gene located on chromosome 7 (7pter-7q22)[10].

The previous reports showed vast differences in CHD mortality rates among different populations and ethnic groups. Some populations still have low rates of CHD mortality, while others have a high incidence[11-13]. This difference has mainly been attributed to existence of varying levels of risk factors in different populations and diabetes is one of these[14]. The very high frequency of CHD among diabetic patients is common and ascribed partly to extensive presence of atherosclerotic plaques[1,15]. The opinion also prevails that diabetes accelerates the mechanism that leads to development of classical atherosclerosis and diabetic subjects with high cholesterol are generally at very high risk of developing CHD[16]. Limited information is available on the influence of known cardiovascular risk factors on stroke mortality in diabetic persons, and the available results are conflicting too[17].

Several researchers have used reverse transcriptase polymerase chain reaction (RT-PCR) to study the response of various stimuli that influence the mRNA levels[18-20], and PDGF-A mRNA[21]. We investigate the role of PDGF-A in various groups of subjects by quantification of PDGF-A mRNA concentration using cRT-PCR and Silver staining of the polyacrylamide gel

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electrophoresis (PAGE). In order to investigate the role of PDGF-A in individuals with or without diabetes and hypercholesterolaemia at risk of CHD, we determined the PDGF-A mRNA in at risk subjects and compared the results with controls. Determination of these risk factors may help to provide a better understanding of the underlying mechanism to adopt appropriate measures to avoid the disease and planning adequate treatment strategy.

MATERIALS AND METHODS

Subjects and samples: Blood samples were collected in 10 ml EDTA tubes from 40 individuals (mean age at investigation 39, range 32-44 years) attending King Khalid University Hospital (KKUH), Riyadh. Subjects were categorized in four groups on the basis of medical history and blood chemistry (n = 10 in each group comprised 5 male and 5 female subjects). Group 1 (controls), healthy non smoking volunteers without a family history of premature CHD with normal glucose and cholesterol levels; group 2, diabetes with normal cholesterol levels; group 3, no diabetes with high cholesterol; and group 4, diabetes and high cholesterol. Medical history, glucose and cholesterol levels were provided by KKUH.

Isolation of total cellular RNA: The RNA was isolated by the method of Chomezynski and Sacchi[22] from peripheral blood mononuclear cells (PBMN) by adding 10 ml of EDTA blood on the Ficoll-Paque (Pharmacia Biotech) followed by centrifugation at 4000g for 20 min at 4°C. The pellet was homogenized with 1 ml of solution D (4 M guanidinium isothiocyanate, 25 mM sodium citrate, pH 5.2, 0.5% *N*-lauryl sarcosine, 0.1 mM DTT and 0.1 mM 2-mercaptoethanol). Sequentially, 0.3ml of 2 M sodium acetate (pH 4.0), 3ml water saturated phenol (pH 4.0) and 0.6ml of choloroform-isoamyl alcohol (49:1) were added to the homogenized pellet. The aqueous phase was separated by centrifugation at 4,000g for 20 min at 4°C, precipitated with 0.6 volume of isopropanol and washed in 75% ethanol. Finally, RNA was dissolved in 20µl DEPC (diethyl-pyrocarbonate) treated sterile deionized (DI) H_2O and stored at -70°C until analyzed. RNA concentration was measured spectrophotometrically at 260 nm by diluting 1 µl of RNA in 249 µl of sterile DI H_2O using GeneQuant-II (Pharmacia Biotech).

cDNA Sysnthesis: GeneAmp®RNA PCR kit (Perkin Elmer) was used for reverse transcription reaction. Briefly, 4 µl of 25 mM $MgCl_2$, 2 µl of 10 X PCR buffer II (500 mM KCl, 100 mM Tris-HCl, pH 8.3), 2 µl of each 10 mM dNTP (dGTP, dATP, dTTP and dCTP), 1 µl of 20 U/µl RNAse inhibitor, 1 µl of 50 U/µl MuLV Reverse Transcriptase, 1 µl of 50 µM random hexamer and 1 µl (5-25 ng) of cellular RNA, 1 µl (10^6 copies) of pAW109 RNA were added in a 0.2 ml PCR tube. The total reaction volume was made up to 20 µl with DEPC treated DI H_2O and incubated at room temperature for 10 min for hexameric primers extension. The reaction tubes were then incubated for reverse transcription using a thermal cylcler (Techne GENIUS, UK) at 42°C for 15 min, denatured at 99°C for 5 min and cooled at 4°C for 5 min.

Polymerase Chain Reaction (PCR): PCR amplify-cation was carried out by using 10 µl of cDNA synthesized by reverse transcription with 2 µl of 25 mM $MgCl_2$, 4 µl of 10X PCR buffer II, 32.75 µl of DEPC treated DI H_2O, 0.5 µl of 15 µM PDGF-A each forward (AW116, 5`-CTGCCATTCGGAGGAAGAG-3`), and reverse primer (AW117, 5`-TTGGCCACCTTGACGC TGCG-3`), and 0.25 µl of 5 U/µl AmpliTaq® DNA polymerase. The PCR reaction was carried out in a final volume of 50 µl in 200 µl thin walled tubes. The reaction was started with denaturation at 95°C for 5 min. The amplification profile involve 25 rounds of denaturation at 94°C for 30 s, primers annealing at 48°C for 45 s, and extension at 72°C for 90 s in a thermal cycler. An additional extension step at 72°C for 7 min was also included at the end of the thermal profile. The reaction tubes were held at 4°C after thermal cycling. PCR products were tested on 1.5% agarose gel electrophoresis by loading 5 µl of the amplicon with the loading dye and visualization on UV after ethidium bromide staining.

Polyacrylamide Gel Electrophoresis (PAGE) and Quantitative Analysis: For quantitative analysis the PCR products were electrophoresed in a Genephor apparatus (Pharmacia Biotech) using ready made 10% polyacrylamide gels (Pharmacia Biotech) at 150V, 25mA and 15W for 2 hours in 0.5 X Tris-phosphate buffer to allow adequate resolution of the target and synthetic internal control. The gel was silver stained by DNA silver staining kit using Hoefer automated gel stainer (Pharmacia Biotech) or a manual procedure[23]. Each pair of signals on PAGE corresponding to the target template mRNA and the cRNA was analyzed by scanning densiotmetry (Hewlett Packard) and quantitated by IQ software (MD ImageQuant Software version 3.22). Quantitation of the target mRNA was performed by comparison with the cRNA internal

standard and expressed per µg of total cellular RNA which leads to linear relationship between RNA concentration and mRNA copy number. The concentration of PDGF-A mRNA was calculated from the relative sample and control peak areas and the known number of molecules of cRNA added to the PCR reaction as described by Powell and Kroon[24].

RESULTS AND DISCUSSION

A number of studies have previously been carried out to investigate the role of risk factors in the development of coronary heart disease (CHD) in diabetic and non diabetic subjects with normal or high levels of cholesterol[16,25,26] but no consistent results have been reported. PDGF-A has also been investigated previously to study its possible role in the CHD[4,27]. However, the data available is conflicting and needs analysis at molecular level using reliable and reproducible method for more convincing results. Therefore, we quantified the expression of PDGF-A mRNA in four different groups of Saudi subjects using cRT-PCR, where a small synthetic RNA (pAW 109) was used as an internal standard (cRNA). The synthetic RNA control (pAW 109) has already been used in several previous studies as internal control[24]. The pAW 109 cRNA contains synthetic PCR primer sites for PDGF-A and a number of other genes thus serves as quantitative internal standard. The PCR primers used are RNA specific i.e. span exons only and therefore do not amplify the intervening sequences. Competitive RT-PCR products were detected by agarose gel electrophoresis and quantitation carried out on PAGE and silver staining. This represents an improvement over a previously published approach where a small synthetic RNA was used as an internal standard and detection system was based on radioactively labeled nucleotides that were incorporated during the amplification step[28].

In the present study, blotting and radiation based detection system was replaced with silver staining, scanning densitometry and quantitation using computer software. The average PDGF-A mRNA copies or number of molecules/µg of cellular RNA are $29 \pm 5 \times 10^4$ (values are mean ± SD) in group one (healthy subjects); $33 \pm 7 \times 10^4$ (group 2); $34 \pm 9 \times 10^4$ (group 3) and $41 \pm 4 \times 10^4$ (group 4). No significant difference in PDGF-A mRNA copies were observed among group 1, group 2 and group 3 whereas a significant increase was noticed in group 4 as compared to control (Fig. 1).

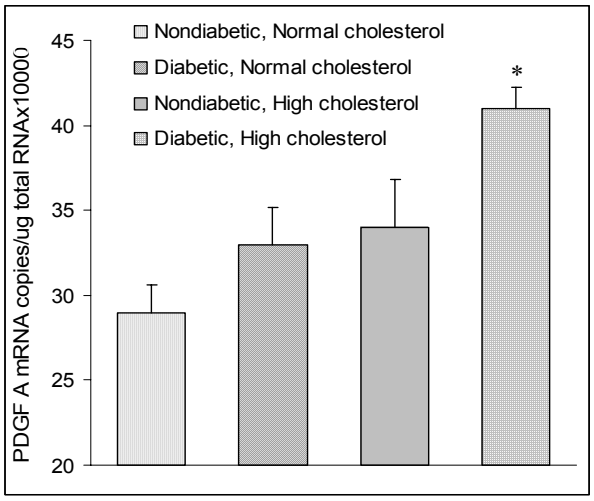

Fig. 1: PDGF-A mRNA copies/µg of total RNA in different groups. Values are mean ± SEM. *P<0.05 versus control (Nondiabetic, Normal cholesterol) group.

The previous reports indicate that PDGF play an important role in the proliferative process of atherosclerosis[29]. The association of high levels of PDGF-A mRNA in diabetic patients with high cholesterol indicates that it can be used as risk factor to predict the development of CHD in these individuals. High copy number of PDFG-A mRNA in diabetic and hypercholesteraemic patients represents some form of activation that might be associated with early stages of CHD.

It is important to establish whether this increase reflects the progress of atherogenesis in patients or PDGF-A mRNA levels are simply an early indicator of the initiation of the process. In either situation, the PDGF-A mRNA levels might represent a valuable non invasive tool to follow individuals at risk of atherosclerosis and a potential parameter for therapeutic intervention. Direct stimulation of PDGF-A gene by metabolites of cholesterol and hyperglycemia could be a simplest explanation, however, it is equally possible that some other factor may also be independently responsible for hypercholesteraemia and diabetes that raised PDGF-A mRNA, or that high cholesterol levels induce changes in cytokine signaling between endothelium, monocytes and/or other cells which initiates PDGF expression. A long term follow up of these subjects may provide more information to establish the role of PDGF-A mRNA as risk factor in the development of CHD.

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