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Assessment of the role of transforming growth factor-beta$_1$, sialic acid, and C-reactive protein in end-stage renal disease premature atherosclerosis

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Abstract

Atherogenesis is accelerated in patients with end stage renal disease. Multiple risk factors for atherosclerosis in patients with end stage renal disease may play an important role in the development and progression of the lesion.

The aim of the present work was to elucidate any possible relationship of transforming growth factor-beta$_1$ (TGF-β_1), C-reactive protein (CRP), and sialic acid to the development of end stage renal disease atherosclerosis. To achieve this goal, this study included 10 end stage renal disease patients on bicarbonate hemodialysis three times weekly for less than 5 years (group I), 10 end stage renal disease patients on conservative treatment (group II), in addition to the 10 healthy volunteers (group III). All studied patients and controls were evaluated clinically and biochemically. Serum TGF-β_1, CRP, and sialic acid were estimated by ELISA, Radial immunodiffusion plates and chemical method, respectively. Echo-Doppler study of the common and the internal carotid arteries was performed for the detection of the late as well as the trivial atherosclerotic changes.

Statistical analysis of the results of the present study showed significantly higher serum levels of TGF-β_1, CRP and sialic acid in patients with end-stage renal disease atherosclerosis whether on hemodialysis or not- compared to end stage renal disease patients without atherosclerosis and healthy volunteers.

From this study we can conclude that elevated serum TGF-β_1 level in ESRD patients with atherosclerosis correlates with the atherogenesis process and probably plays a role in induction of atherosclerosis. Also, serum CRP is elevated in ESRD atherosclerosis and it may be considered as a marker for atherosclerosis in these patients provided that other causes of inflammation are excluded. Serum sialic acid level is also elevated in patients with ESRD atherosclerosis suggesting a possible role in the pathogenesis of the disease.

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Introduction

Cardiac disease is the leading cause of mortality in cardiac patients with end stage renal disease (ERSD) accounting for almost half the deaths in this patient population in the United States [1].

Previously, they hypothesized that atherosclerosis is accelerated in patients with ESRD on maintenance hemodialysis [2]. Postmortem [3] and angiographic [4] studies confirmed that the prevalence of coronary artery disease is increased in dialysis patients compared to patients with similar age without renal impairment.

Transforming growth factor-β (TGF-β) is a member of a large family of growth factors and cytokines that are synthesized by a wide range of cells and therefore are distributed in many different tissues [5]. TGF-β molecules occur in five different isoforms designated TGF-β_1 through TGF-β_5 [6]. Of the five distinct TGF-β isoforms that have been described TGF-β_1, TGF-β_2 and TGF-β_3 have been identified in mammalian tissues and they share 64-82% homology among their amino acid sequence [7].

Various studies have shown that TGF-β_1 plays an important role in a number of diseases: cancer, pulmonary fibrosis [8], osteoporosis [9] and atherogenesis [10].

The role of TGF-β_1 in atherosclerosis is still not well established. "The protective cytokine hypothesis" proposed that TGF-β_1 is the key inhibitor of atherogenesis [10]. On the other hand others proposed

that TGF-β_1 contributes to the pathogenesis of atherosclerosis [11].

C-reactive protein (CRP) is a β-globulin protein with a MW 105kDa. It was originally named after a property of serum that had been obtained from acutely ill patients and which caused the precipitation of a polysaccharide (fraction C) from pneumococcal extracts. CRP binds strongly to certain lipids, particularly phospholipids. It seems that CRP is somehow concerned with the body's response to foreign materials. It is one of the acute phase reactants [12].

CRP may be a factor in the pathogenesis of coronary [13] and carotid [14] atherosclerosis.

Sialic acids are N- or O-acyl derivatives of neuraminic acid. Neuraminic acid is a nine-carbon sugar derived from mannosamine and pyruvate. Sialic acids are constituents of both glycoproteins and gangliosides [15].

The serum concentration of total sialic acid is one recently investigated marker for the atherosclerosis and cardiovascular mortality [16].

The aim of this study was to assess the role of TGF-β1 in ESRD atherosclerosis and to correlate its level with serum levels of sialic acid and CRP.

Subjects

The study was carried out on 30 subjects grouped as follows:

Group I: 10 male patients with end-stage renal disease on regular hemodialysis three weekly for a period less than 5 years with cellulose membrane dialyser of 1.2m^2 surface area and sodium bicarbonate.

Group II: 10 male patients with end-stage renal disease who did not start dialysis (conservative treatment).

Group III: 10 male healthy volunteers within the same age were taken as control group.

Group I and Group II were subdivided into +ve and -ve according to presence (+ve) or absence (-ve) of atherosclerosis.

All patients were males aging from 40 - 50 years.

All diabetic patients, patients with liver cirrhosis, and patients with uncontrolled hypertension or hypertension controlled with β blockers were excluded from the study.

Methods

Estimation of TGF-β_1 by ELISA [17]: Samples were activated then immunoassay was done.

Activation procedure aimed to activate latent TGF-β_1 to immunoreactive TGF-β_1 so it could be detected by immunoreactive TGF-β_1 immunoassay.

2.5 N acetic acid / 10 M urea solution was added to the serum. Neutralization of the samples to pH 7.2-7.6 was done by adding 2.7N NaOH /1M HIPPUS. These activated samples were diluted by 10 folds with the calibrate diluent.

Assay procedure employed the quantitative sandwich enzyme immunoassay technique. TGF-β soluble receptor type II, which bound TGF-β_1, had been pre-coated onto a microplate. Standards and samples were pipetted into the wells and any TGF-β_1 present was bound by the immobilized receptor. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for TGF-β_1 was added to the wells to sandwich the TGF-β_1 immobilized during the first incubation. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells and the color developed in proportion to the amount of TGF-β_1 bound in the initial step. The color development was stopped and the intensity of the color was measured.

Estimation of serum CRP by immunodiffusion plates [18]: The antigen (CRP) was left to diffuse radially from a cylindrical well through an agarose gel containing an appropriate monospecific antibody. Antigen-antibody complexes form a precipitation ring. A linear relationship existed between the square of the ring diameter and the antigen (standard) concentration. Concentration of the CRP in the unknown sample was then determined by measuring the ring diameter produced by that sample and reading off the calibration curve.

Serum protein bound sialic acid was estimated [19]: 5% trichloroacertic acid (TCA) was added slowly with shaking to the serum samples. Tubes were boiled in a boiling water bath for 15 minutes. Filtration was done. Clear filtrate was put into each of two tubes. Diphenylamine (DPA) reagent was placed into one of each pair of the tubes and acid mixture containing no DPA was placed into the other tube. (The acid mixture was composed of Glacial acetic acid and concentrated sulfuric acid). Boiling in water bath for 30 minutes was done.

Calculation of the concentrations was done from the difference in the optical densities (OD) in the presence and absence of DPA in order to correct the non-specific development of color.

$$\text{Sialic acid (mg/dl)} = \frac{\text{(OD test + DPA) - (OD test - DPA)}}{\text{(OD standard + DPA) - (OD standard - DPA)}} \times 20$$

Serum cholesterol, triglycerides, low-density lipoprotein-cholesterol and high-density lipoprotein cholesterol were measured [20].

Statistical analysis

ANOVA test (F-test) was used for the evaluation of statistical significance. Differences were considered significant at $P < 0.05$ levels. Values were expressed as mean (X) ± standard deviation (SD).

Results

Carotid Doppler US findings and clinical data of studied patients are given in table 1.

Table 1. Carotid doppler findings of clinical data

	G I (n=10)	*G II (n=10)*	*G III (n=10)*
Carotid doppler findings	4 (+ve)	3 (+ve)	-
• Lumen-intima interface/media-			

adventitia interface >0.5mm distance.			
• Focal widening of wall.			
• Plaques			
Clinical data			
• Effort angina.	3	2	-
• CVS.	1	2	-
• TIA.	1	1	-
• Intermittent claudication.	-	1	-

CVS: Cerebrovascular stroke
TIA: Transient ischemic attack

Patients with carotid atherosclerosis (+ve) in both ESRD groups (GI and GII), showed significantly higher levels of serum TGF -β_1 than that of patients without carotid atherosclerosis (-ve), and of control group (GIII). [F=5.98, P<0.01]. (table 2)

Patients with carotid atherosclerosis (+ve) in both ESRD groups (GI and GII), showed a significantly higher levels of serum sialic acid than that of ESRD patients without carotid atherosclerosis (-ve), and of control group (GIII). [F=5.21, P<0.05]. (table 2)

Patients with carotid atherosclerosis (+ve) in both ESRD groups (GI and GII), showed significantly higher levels of CRP than that of patients without atherosclerosis (-ve), and of the control group (GIII). [F=4.21, P<0.05]. (table 2)

A significant positive correlation was found between serum TGF-β_1 and CRP in both GI and GII (r = 0.9, P < 0.01) (Fig 1, 2), while no significant correlation was found between TGF-β_1 and sialic acid in both groups. Taking both ESRD patients together (GI and GII), a significant correlation was found between TGF-β_1 and both serum sialic acid (r=0.71, P< 0.05) (Fig 3), and serum CRP (r=0.74, P < 0.05).

Table 2. Serum TGFβ_1, sialic acid, and CRP in ESRD patients with atherosclerosis (+ve), without atherosclerosis (-ve) and in control group III

	ESRD (GpI & II) +ve "n=7"	-ve "n=13"	*Control Gp. III "n=10"*	*F value*	*P*
TGFβ_1 (ng/mL)					
Range	50.8-75.3	12.2-35.3	15.6-23.2	5.98	<0.01*
Mean ± S.D.	60.5±8.6	25.7±5.9	19.4±2.9		
Sialic acid (mg/dl)					
Range	36.7-78.6	24.2-132.2	21.3-27.9	5.21	<0.05*
Mean ± S.D.	53.4±15.3	64.1±29.5	24.8±2.3		
C-reactive protein (mg/L)					
Range	39.5-61	4.3-14.8	4.3-14.8	4.21	<0.05*
Mean ± S.D.	51.3±6.5	7.4±3.3	6.5±3.4		

* Statistically significant

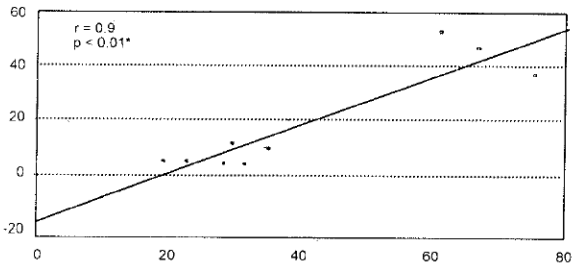

Fig 1. Correlation between serum TGF-β_1 levels and CRP levels in the dialysis patients (GI)

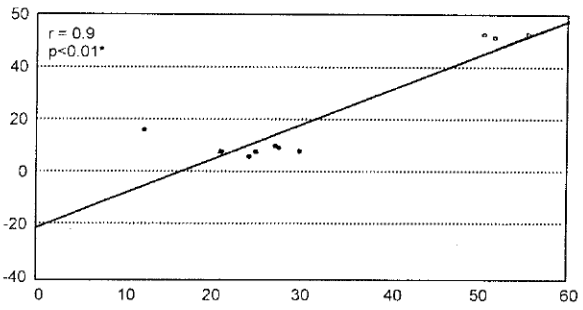

Fig 2. Correlation between serum TGF-β_1 levels and CRP levels in the non-dialysis patients (GII)

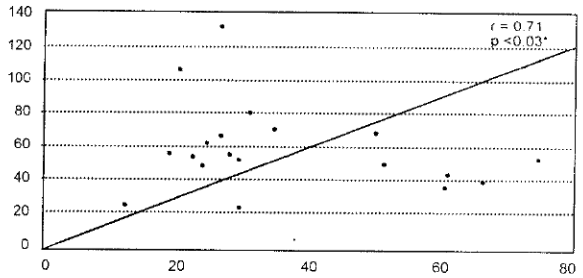

Fig 3. Correlation between serum TGF-β_1 levels and serum sialic acid levels in ESRD (GI and GII)

Discussion

Cerebrovascular disease is the single best predictor of mortality in patients with ESRD, as it accounts for almost 50 percent of deaths [21]. The pathogenesis of ESRD atherosclerosis is not fully understood. Its high prevalence, however, appears to result from the multiplicity of risk factors for atherosclerosis in patients with ESRD [22].

The present study measured serum TGF-β_1 in ESRD patients both on dialysis (GI) and on conservative treatment (GII). Positive patients for atherosclerosis

within each group (as proved by carotid Doppler ultrasonography) were compared with the negative ones within the same group (GI and GII) and with healthy volunteers (GIII).

There were statistically elevated serum TGF-β_1 levels in patients with atherosclerosis among the dialysis and non-dialysis groups as compared to all ESRD patients without atherosclerosis and healthy volunteers.

This result was consistent with that of Blann et al (1996) where they reported increased levels of TGF-β_1 receptor CD105 in patients with peripheral vascular disease [23].

Stephen et al (1998) [24] suggested that TGF-β_1 contributes to the progression of atherosclerosis by stimulating proteoglycan synthesis.

Bobik et al (1999) [11] reported that TGF-β_1 contributes to the pathogenesis of atherosclerosis by stimulating proteoglycan biosynthesis in human arterial smooth muscle cells which interacts strongly with lipoproteins slowing their transit in the vessel wall and thereby increasing the possibility for lipoprotein deposition [25]. Also modifications of these lipoproteins by peroxidative processes frequently produces end products, including 4-hydroxy-2,3-nonenal that up-regulates TGF-β_1 expression in macrophages [26] inducing them to produce urokinase plasminogen activator and subsequently plasmin [27] an activator of metalloproteinase [28] systems implicated in plaque instability and rupture [29].

On the other hand, some investigators reported that TGF-β_1 inhibits atherogenesis [10]. Active TGF-β_1 is an inhibitor of vascular smooth muscle cell migration and proliferation and therefore prevents the development of atherosclerosis. Furthermore, it is thought that TGF-β_1 maintains the endothelial function by suppressing leukocyte adhesion to the endothelial cells both in the presence and absence of inflammatory cytokines [30].

Also Nikol et al (1992) [31] stated that TGF-β_1 is the most potent stimulator of connective tissue formation, so it alters the deformability of the atherosclerotic lesion by preventing fissuring, cracking or ulceration of the plaque that lead to hemorrhage and set the stage for formation of terminal thrombus.

The present study showed a statistical significant elevation of serum protein bound sialic acid level in patients with atherosclerosis among the dialysis and non-dialysis groups as compared to ESRD patients without atherosclerosis and to healthy persons.

The findings of this study appear to conflict with the findings of Salamone et al (1998) [32] who concluded that the serum sialic acid is not a useful marker of the disease severity in atherosclerosis. Also results of this study did not meet the findings of Wu et al (1999) [33] who found no significant correlation between the plasma sialic acid level and the coronary artery atheromatous load as judged by semi subjective scoring system and concluded that the relationship between sialic acid and atherosclerosis is not straight forward being mediated most probably by plaque activity or indirectly via other risk factors.

On the other hand Lindberg et al (1991) [34] reported that serum sialic acid concentration is associated with the death from cardiovascular disease in general and from coronary heart disease in particular [35]. Watts et al (1995) [36] postulated that serum sialic acid might be an indicator of coronary heart disease progression.

Lindberg et al (1999) [37] found that the serum level of total sialic acid was elevated in patients with carotid atherosclerosis together with the serum levels of orosomucoid, haptoglobin and α_1 antitrypsin (glycoproteins which together incorporate about one half of the serum total sialic acid). There was also a strong bivariate relationship between serum total sialic acid and each of the glycoproteins rendering them responsible for elevation of the former.

The major part of the serum sialic acid is carried on glycoproteins of which several are acute phase proteins [38]. The level of serum glycoproteins has been shown to correlate with coronary atherosclerosis, severity of the atherosclerosis, and with atherosclerosis related conditions such as peripheral vascular disease [39].

The present study measured CRP in ESRD patients whether on dialysis (GI) or not on dialysis (GII). Positive patients for the atherosclerosis within each group were compared with the negative ones within the same group and with healthy volunteers (GIII).

There was a statistically significant elevated CRP level in patients with atherosclerosis among the dialysis population, non-dialysis patients compared to ESRD patients without atherosclerosis and healthy volunteers.

The European Concerted Action on Thrombosis and Disabilities Angina Pectoris Study [40] found a significant correlation between the CRP concentration within the normal range and the long-term cardiovascular risks.

Palosou et al (1992) [41] concluded that most patients with unstable angina have increased levels of serum CRP for months after symptoms develop independent of the severity of their atherosclerosis.

Zhang et al (1999) [13] postulated that CRP plays an important role in the development of atherosclerosis.

On the other hand, results of this study seemed to conflict with the results of Canova et al (1999) [42] who concluded that CRP has no clinically useful prognostic value in patients with acute cerebrovascular events. However, this study differs than that of Canova et al being performed on ESRD patients who are exposed to different risk factors for atherosclerosis than those with normal kidney functions. Some of these risk factors may be more stressful and injurious to the arterial wall than the ordinary risk factors causing more intense inflammation and thus more elevation of the serum CRP. CRP concentration could be related to the pathogenesis of atherosclerosis via arterial inflammation resulting from conventional risk factors or that the raised CRP concentration may result from arterial wall inflammation associated with the atherosclerosis itself [43].

The present study showed a significant correlation between TGF-β_1, serum protein bound sialic acid and CRP in ESRD patients. In ESRD patients, TGF-β_1 reduces atherosclerosis-, which is a systemic inflammatory reaction-, which in turn increases the serum sialoglycoproteins level (most of them are acute phase reactants) raising in turn the serum sialic acid and CRP levels.

From this study we can conclude that elevated serum TGF-β_1 level in ESRD patients with atherosclerosis

correlates with the atherogenesis process and probably plays a role in induction of atherosclerosis. Also, serum CRP is elevated in ESRD atherosclerosis and it may be considered as a marker for atherosclerosis in these patients provided that other causes of inflammation are excluded. Serum Sialic acid level is also elevated in patients with ESRD atherosclerosis suggesting a possible role in the pathogenesis of the disease. Finally, carotid Doppler ultrasonography is effective as a non-invasive procedure for screening of atherosclerosis in ESRD patients.

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