ISSN: 0975-833X

Available online at http://www.journalcra.com

International Journal of Current Research
Vol. 10, Issue, 04, pp.67514-67518, April, 2018

INTERNATIONAL JOURNAL OF CURRENT RESEARCH

RESEARCH ARTICLE

ASSOCIATION OF ELEVATED LEVEL OF PLASMA FIBRINOGEN WITH LOW GRADE CHRONIC INFLAMMATION IN PATHOGENESIS IN DIABETES MELLITUS

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ARTICLE INFO

Article History:
Received 17th January, 2018
Received in revised form
05th February, 2018
Accepted 28th March, 2018
Published online 30th April, 2018

Key words:
Diabetes mellitus, fibrinogen,
Innate immunity,
Chronic inflammation.

ABSTRACT

Aim: Fibrinogen is an inflammatory marker which is an acute phase protein, plays a key role in the pathogenesis of atherosclerosis and complication of atherothombosis disease. Pathogenesis of type 2 diabetes mellitus is closely related with acute phase response which is predominately mediated by cytokine. By estimating circulating fibrinogen in type 2 (T-2) and type1 (T-1) diabetic patients, I tried to establish this hypothesis.

Method: Freshly diagnosed twelve T-1 cases, twenty-five T-2 cases and twenty-five Type-2 cases under oral hypoglycemic agent for at least 5 years were selected and were estimated the level of fibrinogen. Thirty normal controls were also selected.

Result: Freshly diagnosed T-1 and Type-2 cases showed significantly higher levels of the fibrinogen in compare to control. T-2 cases had slightly elevated values of fibrinogen in compared to the T-1 cases. There are no significant change of fibrinogen level is found in T-2 cases after treating by oral hypoglycemic drugs in compare of T-2 cases.

Conclusion: By evaluating the plasma level of fibrinogen in different categories it can be postulated that a low grade inflammatory process is definitely implicated in the pathogenesis of both type 1 and type 2 cases. This line of pathological basis should be further explored for diagnosis, management and follow up.

Citation: Shamim Shaikh Mohiuddin and Poornima Manjrekar, 2018. "Association of elevated level of plasma fibrinogen with low grade chronic inflammation in pathogenesis in diabetes mellitus", *International Journal of Current Research*, 10, (04), 67514-67518.

INTRODUCTION

Diabetes mellitus is a aggregation of metabolic disorder characterized by elevated level of blood sugar resulting from either in defect of insulin secretion, insulin action or both.Even in the presence of intensive glycemic control diabetes mellitus is seen with association with an elevated risk of cardiovascular disease. Both diabetes mellitus and insulin resistance cause a combination of dysfunctions of endothelium of microvasculature, leading to diminish the antiatherogenic role of the vascular endothelium. Hence, in these patients endothelial dysfunction may be a critical early target for preventing atherosclerosis and other cardiovascular complications (Hadi and Suwaidi, 2007). Fibrinogen is the one of the most important coagulation protein in blood which is also acted for the precursor of fibrin and blood viscosity and platelet aggregation is mainly determined by concentration of plasma fibrinogen level.

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Because fibrinogen level can be reduced considerably by life style interventions that also affect levels of established risk factors (such as regular exercise, smoking cessation, and moderate alcohol consumption) there is possibility that measurement (or modification) of fibrinogen may help in disease prediction or prevention (Danish *et al*., 2005). Fibrinogen is a soluble plasma glycoprotein consisting of three nonidentical pairs of polypeptide chains (Aα, Bβ, γ) covalently linked by disulfide bonds. It has a molecular weight of 340000 and half-life of 2.5 days. The Bβ and γ chains contain asparagine linked complex oligosaccharides. All three chains are synthesized in liver. The three structural genes involved are on the same chromosome and their expressions are coordinately regulated in humans. The amino terminal regions of the six chains are held in close proximity by number of disulfide bonds, while the carboxyterminal regions are spread apart, giving rise to a highly asymmetric elongated molecule. The A and B portions of the Aα and Bβ chains are designed as fibrinopeptide A (FPA) and B (FPB) respectively. These are situated at the amino terminal ends and the chains bear excess negative charges as a result of presence of aspartate and glutamate residue as well as an unusual tyrosine O-sulphate in

FPB. These negative charges contribute to the solubility of fibrinogen in plasma and also serve to prevent aggregation by causing electrostatic repulsion between fibrinogen molecules (Murray *et al*., 2002). One of the most important marker of inflammation is plasma fibrinogen and is also a potent prothrombotic factor responding to injury of endothelium tissues. It may an indicator of an inflammatory vascular changes and endothelial dysfunction. Fibrinogen as an acute phase protein plays a key role in the pathogenesis of atherosclerosis and complication of atherothombosis disease. Increased fibrinogen level is considered as an important atherosclerosis risk factor. Patient with type 2diabetes frequently have increased fibrinogen levels (Doweik *et al*., 2003). The entire coagulation cascade is dysfunctional in diabetes.

Increased level of fibrinogen and plasminogen activator inhibitor favors both thrombosis and defective dissolution of clot once formed. Elevated fibrinogen levels in high risk patients with peripheral arterial disease indicate an increased risk of poor outcome, particularly for fatal cardiovascular complications (Crook *et al*., 1993). Over the past few years, attention has been focused on fibrinogen, regarding its elevated role in the pathogenesis of cardiovascular risks in diabetic patients. Many extensive research even shown that thrombotic (smoking, low fruit/vegetable intake, fibrinogen and homocysteine) as well as atherosclerotic (hypertension, high fat diet, dyslipidemia) were important risk factors in premature coronary heart disease (CHD) (Panwar and Gupta, 2011)).Together with other haemostatic factors, fibrinogen may promote atherosclerotic changes and thrombosis through effects shown on platelet aggregation in vitro, blood viscosity and formation of foam cell. But till now, not many studies have been established a clear relationship between plasma fibrinogen levels as a low grade inflammatory markers and type 2 diabetes mellitus. The high prevalence as well as incidence of conventional coronary risk factors in patients with type 2 diabetes mellitus not able to establish the proper explanation of increased cardiovascular-related morbidity and mortality in these patients. Fibrinogenmay have a role in this excess risk. A whole plentitude of newly emergence questions related to plasma fibrinogen in association to type 2 diabetes mellitus has opened by this study. Not so many extensive studies have been undertaken in the past on this particular subject, and most of those which were done, failed to arrive at a substantial definite conclusion. This leaves the area of study even more intriguing and fascinating than ever.

Fibrinogen is widely identified as a major independent risk factor of cardiovascular disease. It is acting as one of the very important acute phase proteins and a positive marker of inflammation which are considered as predictive risk of myocardial infarction, stroke, peripheral arterial disease and sudden cardiac death. Acute phase reaction is a general reaction to inflammation, comparable to the increase in temperature or leukocyte count and is not specific for any given disease. A small protein known as Leucocytic Endogenous Mediator (LEM) which is released from the site of injury probably triggers all these changes (Paul and Ridkar, 2003). Plasma fibrinogen is also an important component of the cascade of coagulation as well as a major determination factor of viscosity of blood and flow of blood through vessels. Increasing proportion of suggestion from different epidemiological studies suggest that elevated plasma fibrinogen levels are closely related with an increased risk of cardiovascular disorders including ischemic heart disease, stroke and others cardiovascular complication like thromboembolism (Meade *et al*., 1986; Wilhelmsen *et al*., 1984). It has been shown that high fibrinogen concentration enhances therisk of cardiovascular disease in diabetic patients (Christe *et al*., 1984; Kannel *et al*.,1990; Ganda *et al*., 1992). Insulin acutely increases fibrinogen production in an individual ith type-2 diabetes but not in individual without diabetes. There is significant correlation between fibrinogen level and fasting blood sugar level (FBS) (Pierpaolo *et al*., 1991).

Aims and Objectives

The aims of the present study are

- To detect the elevation of plasma level of fibrinogen as an inflammatory markers(acute phase reactant), if any, in newly diagnosed untreated type 1 diabetes mellitus patients, in newly diagnosed untreated type 2 diabetes mellitus patients and in patient of type 2 diabetes mellitus under treatment for at least five years.
- Compare the level of fibrinogen in newly diagnosed untreated type 2 diabetes mellitus patient with type 2 diabetes mellitus patients under treatment for at least five years.
- Compare the level of fibrinogen in newly diagnosed untreated type 1 diabetes mellitus with newly diagnosed untreated type 2 diabetes mellitus patients.

MATERIALS AND METHODS

Study design

Following four groups of subjects were selected for present study

- Twelve newly diagnosed untreated type 1 diabetes mellitus patient
- Twenty-five newly diagnosed untreated type 2 diabetes mellitus patients within the age limit of 30-60 years
- Twenty-five type 2 diabetes mellitus patients who are under treatment of oral hypoglycemic drugs for at least 5 yrs between the age limit of 30-60 yrs.
- Thirty nondiabetic healthy controls.

Height and weight of all subjects were recorded and body mass index was calculated. None of the ninety two volunteers were alcoholics or smokers. The participants did not suffer from chronic inflammatory diseases like asthma, chronic bronchitis, and rheumatoid arthritis as was ascertained by clinical history. Informed consent was taken from the individual subjects prior to blood collection. The study was undertaken in Kasturba Medical College, Mangalore, India and approved by institutional ethical committee.

Fibrinogen assay

Principle

Fibrinogen assay in plasma was carried out by Biuret method (Varley *et al*., 1991).

Fibrinogen present in the plasma is converted to fibrin in presence of calcium chloride. The fibrin clot formed is collected and then digested with NaOH. Protein content of the clot is determined using a red filter.

Procedure

- 0.5 ml of plasma was mixed with 14 ml of distilled water and 0.5 ml of 2.5% calcium chloride solution in a small beaker and incubated at 37ºC until a clot was formed. Then a glass rod was rotated to collect the clot on to it.
- The rod was pressed against the side of the beaker to squeeze out any solution and to compress the clot. Care was taken to pick up any small piece of clot on the rod, which may have become detached and was dried by pressing carefully against a filter paper. Then it was transferred into a test tube into which the digestion was to be carried out.
- After that, the clot was digested with 0.5 ml of 0.1N NaOH in a boiling water bath. After cooling, 3.5 ml of working Biuret reagent was added to the tube
- The OD of the blue color developed was read at 555nm after standing the tube in a water bath at 37ºC for 5 minutes.
- 0.5 ml of standard protein solution (800mg/dl) and 0.5 ml of distilled water as blank were treated similarly.

Calculation

$$\text{Concentration of fibrinogen (mg/dl)} = \frac{\text{Absorption of sample}}{\text{Absorption of standard}} \times 800$$

Statistics

The data was analyzed by the students' t test and the ANOVA test. Pearson's coefficient was applied for correlational analysis.

RESULTS

- Group I = Type 1 diabetes mellitus patient (newly diagnosed)
- Group II = Type 2 diabetes mellitus patient (newly diagnosed)
- Group III = Type 2 diabetes mellitus patient (under treatment for at least 5 years)
- Group IV = Control
- *denoted significant value
- n = number of subjects
- SD = Standard Deviation
- BMI= Body Mass Index
- RBS= Random Blood Sugar

DISCUSSION

The aim of this study was to examine inflammation as a pathogenetic cause in type 1 and type 2 diabetes mellitus. In the twelve newly diagnosed type 1 patients, the level of fibrinogen was found to be significantly increased as compared to control and finding is considered as statistically significant as p value is found as < 0.0001* (Table 3). Previous reports on the fibrinogen levels in Type 1 diabetes are contradictory. Crooke MA *et al* (Crook *et al*., 1993) has shown that serum sialic acid and fibrinogen are not elevated in type 1 diabetes. Gomes *et al.* (Gomes *et al*., 2003) reported increased level fibrinogen in Type 1 patients. Increased fibrinogen levels, factor VII and whole blood viscosity was also found by John AD Elia *et al.* (Elia *et al*., 2001). Similar results are reported by Defeo *et al.* (Defeo *et al*., 1993). In our study, an increase fibrinogen level of fibrinogen was found in type 1 as well as type 2 diabetic patients. Twenty-five type 2 newly diagnosed patients showed increased levels of fibrinogen in compare to control (Table-3) as denoted by significant p value (< 0.0001*). The findings were in agreement with most of the authors who worked with fibrinogen in type 2 diabetes. (Pickup, 2004; Mc Millan, 1989; Festa *et al*., 2002). The role of chronic low grade inflammation in the pathogenesis of type 2 diabetes seems possible beyond doubt. At the same time its role in type 1 diabetes cannot be totally ruled out. The course of the disease and resulting complications are similar in both type 1 and type 2 diabetes. The most dreaded complication being that of development of atherosclerosis resulting in cardiovascular diseases. Fibrinogen is identified as an independent risk factor in the development of ischemic heart diseases (Ernst and Resch, 1993). Irrespective of the patients being type 1 or type 2, the risk of developing atherosclerosis remain the same. Hence there must be some mechanism which links the pathogenicity of type 1 and type 2 diabetes mellitus. Barrazzani R *et al* (Barrazzani *et al*., 2003) infused insulin to non-diabetics, type 1 and type 2 diabetics and studied its role in fibrinogen production. Insulin replacement activity suppressed fibrinogen production in non-diabetics and type 1 diabetic individuals.

Table 1. The anthropometric data of the subjects participated in the study are presented in Table 1

	Group I(n=12) (Mean ± SD)	Group II (n=25) (Mean ± SD)	Group III (n=25) (Mean ± SD)	Group IV(n=30) (Mean ± SD)
Age (yrs)	18.33 ± 7.64	48.22 ± 7.11	51.32 ± 7.56	44.97 ± 15.06
BMI	19.50 ± 1.23	24.03 ± 1.46	24.20 ± 2.40	21.75 ± 2.27
RBS	338.25 ± 50.97	193.26 ± 35.30	93.61 ± 33.65	94.20 ± 7.00

Table 2. The compare of mean value of fibrinogen in groups in Table 2

	Group I (Mean ± SD)	Group II (Mean ± SD)	Group III (Mean ± SD)	Group IV (Mean ± SD)
Fibrinogen(mg/dl)	434.65 ± 46.36	572.25 ± 82.26	581.74 ± 79.09	335.34 ± 42.19

Table 3. Comparison of level of plasma fibrinogen (mg/dl) between different groups in Table-3

Comparison between groups	Level(mg/dl)	Level(mg/dl)	p value
Comparison between Group I and Group IV	434.65 ± 46.36 (I)	335.34 ± 42.19(IV)	< 0.0001*
Comparison between Group II and Group IV	572.25 ± 82.26(II)	335.34 ± 42.19(IV)	< 0.0001*
Comparison between Group III and Group IV	581.74 ± 79.09(III)	335.95 ± 42.19(IV)	< 0.0001*
Comparison between Group I and Group II	434.65 ± 46.36(I)	572.25 ± 82.26(II)	< 0.0001*
Comparison between Group II and Group III	572.25 ± 82.26(II)	581.74 ± 79.09(III)	0.682

(p value < 0.05 is considered significant)

Fibrinogen production and its plasma concentration increased in insulin resistant type 2 diabetics when euglycemia and euaminoaciduria were maintained. They postulated that an altered response to insulin causes hyperfibrinogenemia in type 2 diabetic patients. If this hypothesis holds well, it doesn't explain hyperfibrinogenemia in type 1 diabetics where the basic pathology is insulin deficiency. Hence there must be some other factors which stimulate increased fibrinogen synthesis in type 1 patients contributing to cardiovascular disease risk. An insulin resistance syndrome score (Williams *et al*., 2000) was developed based on clinical risk factor in patient with type 1 diabetes and validated using euglycemic-hyperinsulinemic clamp studies. Fibrinogen levels were significantly associated with this insulin resistance syndrome score. This may explain high fibrinogen level in type 1 diabetes. But it still does not answer the above findings since the type subjects in this study were newly diagnosed. Hence the mechanism of increased fibrinogen synthesis needs to be proved further. The values of fibrinogen when compared between the untreated type 1 and type 2 patients reveal a significant increase in type 2 patients (Table-3). The mean random blood sugar (RBS) values in group 1(Type 1 newly diagnosed diabetes) patients was 338.25 ± 50.97 mg/dl and that of group II (Type 2 newly diagnosed diabetics) was 193.26 ± 35.30 mg/dl (Table-1).

In spite of this huge difference, the inflammatory markers like fibrinogen levels were higher in the type 2 patients (Table-3) which go to prove that the glycemic status doesn't influence the inflammatory markers. This is in accordance with previous findings (Sriharan *et al*., 2002) Evidence is available to say that inflammatory markers are elevated well before the clinical manifestation of hyperglycemia (Engstrom *et al*., 2003; Schmidt *et al*., 1999; Duncan *et al*., 1999; Pradhan *et al*., 2001). This also gives credence to the thought that activation of innate immunity is not related to hyperglycemia. But research has shown that decreasing plasma glucose levels decrease the concentration of acute phase reactants (Gavella *et al*., 2003). Also 2 hrs post load glucose values showed positive correlation with the inflammatory markers in few studies (Sriharan *et al*., 2002).

Having demonstrated that there is an inflammatory process going on in type 2 diabetes, we next thought of estimating the level of fibrinogen as inflammatory markers in patients on treatment(for at least five years) with oral hypoglycemic drugs. Many of the drugs have been shown to have anti-inflammatory effects. HMG-CoA reductase is inhibited by statin group of drugs and this group of drugs used to prevent atherosclerosis by diminishing the deposition of LDL particles rich in cholesterol and phospholipids in macrophages and smooth muscle cells (Manford., 2001) and also seen to inhibit the acute phase response. Freeman DJ *et al* (Freeman *et al*., 2001) showed that statin therapy also prevent diabetes mellitus. Angiotensin Converting Enzyme Inhibitors (ACE inhibitors) are also known to decrease insulin resistance in either type 1 or type 2 diabetic patients with concomitant hypertension (Pollare *et al*., 1989). Torlone E *et al* demonstrated that ACE inhibitors improved glycemic control in patients with arterial hypertension and type 2 diabetes (Torlone *et al*., 1993). Insulin used to have potent anti-inflammatory property (Campus and Baumann., 1992). Insulin was found to be a rapid nonspecific and dose dependent inhibitors of the cytokine and glucocorticoids stimulation of acute phase protein, gene expression and exerted effect at the transcriptional levels. Insulin inhibition applied to all cell cytokines tested but to various degrees depending upon the particular acute phase gene (Campus and Baumann, 1992). In this study, out of the twenty-five type 2 diabetic patients on treatment for at least 5 yrs(Group III), seven patients were on Glitazone, eight patients were in sulfonylurea-metformin combination, six patients were on sulfonylurea alone, two were on metformin alone and two were on glitazone-metformin combination and. When compared with newly diagnosed untreated group (Group II) no significant difference was found in the level of fibrinogen. The RBS values were quite lower to those of untreated group (93.62 ± 33.65 and 193.26 ± 35.30). No change in fibrinogen values suggest multiple pathway involvement that are poorly understood. For centuries we have known of the existence of two types of diabetes; the type 1, where the basic defect is an absolute deficiency of insulin due to an autoimmune destruction of the β cells and the type 2 diabetes, where the underlying pathology is decreased secretion of insulin or an increased resistance to the action of insulin by the insulin sensitive tissues. Then come the era of finding newer and newer mechanisms involved it the pathology.

One that received wide acceptance and paved way for further research is the role low grade chronic inflammatory response and activated innate immunity in the pathogenesis of type 2 and probably type 1 diabetes. In continuation with the ongoing research world over we tried to examine whether this hypothesis holds true by determination of level of fibrinogen as an inflammatory marker and whether the levels will be elevated or not. We can say with conviction that there is an activated innate immunity and a resultant increase in acute phase proteins in newly diagnosed type 2 diabetes. Fibrinogen as an acute phase proteins was also significantly higher in the newly diagnosed type 1 patient also. Irrespective of oral hypoglycemic drug used for the treatment, treated group showed significantly lower acute phase protein levels comparable to the control group except fibrinogen levels. What causes the innate immunity activation? Is it a cause or an effect of diabetes? What is the role of hyperglycemia? Is it associated with other complications of diabetes and if so, how? – are a few of the question which need to be addressed by intensive research. The mechanisms could be multifactorial and complex. A few hypothesis have been postulated which are still wanting.

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