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Research Paper

Study of α-1 Antitrypsin Serum and its Effects on Chronic Inflammation in Diabete Patients

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Abstract

Pathogenesis of type 2 as well as type1diabetes mellitus is observed to be closely associated with acute phase response which is predominately cytokine-mediated. In this present study I try to test this hypothesis by estimating circulating α1 antitrypsin (AAT) in freshly diagnosed type 1 (T-1), freshly diagnosed type 2(T-2) as well as type 2 diabetic patients under oral hypoglycemic drugs for duration of at least five years. AAT is considered as a prominent member of acute phase protein and very important tools for diagnosis for low grade chronic inflammatory reaction. Thirty normal controls to match the age and sex of the test groups were also studied. The level of this parameter was also correlated with their random plasma glucose values and BMI. The value of AAT significantly elevated in the T-2 patients ($p<.00001$) in comparison with the controls. In case of T-1 patients the level of AAT found mildly elevate as p value has marginally significant value of 0.0002. Interestingly in either of the types, no correlation was found with the degree of hyperglycemia or BMI. By the above results and findings is can be definitely postulated that a low grade inflammatory process is surely associated in the pathogenesis of type 2 diabetes. But for type 1 diabetic patient the result is contradictory. This can be further explored for further diagnosis, management and follow up.

Keywords: α1 antitrypsin; acute phase proteins; acute phase reactants; low grade chronic inflammation; diabetes mellitus;

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1. Introduction

Alpha 1-Antitrypsin (AAT) is an acute phase reactant with antiprotease activity (1) which act as a serine protease inhibitor and recently has been focused for prevention for development of Type 1 diabetes, for prolongation of isl et al lograft survival and for inhibition of apoptosis of pancreatic B-cell in vivo (2).In addition to its antiprotease activity, AAT has likewise been shown to have other biological effects, including the ability to modulate both inflammation and apoptosis(3,4).AAT was isolated and described in 1955. Interest in this glycoprotein has centered on its genetic polymorphism and the disease associated with its biological function as a protease inhibitor. AAT is a single chain protein of 394 amino acids which contains three oligosaccharide chains. It is the major component (>90%) of the α-1 fraction of human plasma. It has a molecular weight of ~55000, pI of 4.8 and contains 10-12% carbohydrate. It is synthesized by hepatocytes and macrophages and is the principal serine protease inhibitors of human plasma (5). The name AAT is almost a misnomer since AAT is relatively inactive towards trypsin, although responsible for 90% of serum antitrypsin activity. As a protease inhibitor AAT acts against chymotrypsin, kallikrein, rennin, urokinase, plasmin and possibly thrombin but greater clinical significance regarding inhibitory activity is directed against neutrophil elastase and collagenase by formation of complex with them. The inhibitory activity of AAT is maximal at the neutral to slightly alkaline pH of blood. At pH 4.5 its activity is negligible. When AAT acts as an inhibitor of intestinal enzyme such as trypsin, this pH dependency is less. It is more when it plays in the respiratory tract (6). AAT has significant microheterogenecity (7). Depending on buffer pH and type of support of electrophoresis, a number of

bands may be seen. At least 75 polymorphic forms occur, many of which can be separated by electrophoresis. Even after sialidase treatment, heterogenecity remains unaltered which indicate that AAT phenotypes are probably not due to difference in sialic acid moieties. Typing of multiple forms is empirically based on differences in electrophoretic nobilities (8). Some 33 allotypes have been described. About 95% genotype is PiMM (homozygotic for M protease inhibitor) and is called M protein. Two other proteins Z and S are found in genotypes PiZZ, PiSZ, PiSS, PiMZ and PiMS. Z and S protein differ from one another and from M protein by only one amino acid residue. Circulating level of M protein is directly related to ability to inhibit protease. If normal activity in M phenotype is taken as 100%, the level of activity in ZZ is 15%, in SS 60%, in MZ
57.5% and in MS 80%. A rare null genotype (Pi-) produce no AAT properties at all (9).The function of AAT is to neutralize lysosomal elastase released upon phagocytosis of particles by polymorphonuclear leukocytes. Being a small molecule AAT can pass from capillaries into tissue fluid, bind protease and pass back into intravascular fluid. It may even transfer bound protease to α-2 macroglobulin, which because of its large size cannot leave the intravascular compartment. By this mechanism protease is rapidly transported to the reticuloendothelial system for rapid degradation.

There is increasing evidence that an ongoing cytokine induced acute phase response which is sometimes called low grade inflammation, but part of a widespread activation of the innate immune system, is closely involved in the pathogenesis of type 2 diabetes mellitus and associated complications such as dyslipidemia and atherosclerosis. Elevated circulating inflammatory markers such as C-reactive protein and interleukin-6 (IL-6) predict the development of type 2 diabetes and several drugs with anti-inflammatory properties such as aspirin and thiazolidinedions lower both acute phase reactants and glycemia and possibly decreased the development of type 2 diabetes. Among the risk factors for type 2 diabetes, which is also known to be associated with active innate immunity, are age, inactivity, certain dietary components, smoking, psychological stress and low birth weight. Other features of type 2 diabetes such as fatigue, sleep disturbance and depression are likely to be at least partly due to hypercytokinemia and activated innate immunity (10).

A major uncertainty is whether hyperglycemia is a main determinant of the inflammation in type 2 diabetes mellitus- there is evidence for and against. Cross sectional studies of type 2 diabetes mellitus show that acute phase reactant like C reactive protein (CRP) and Interleukin-6 (IL-6) are significantly correlated with blood glucose concentration or glycated hemoglobin percentage (11). Lowering of blood glucose levels in type 2 diabetic patient are accompanied by reduced levels of inflammatory markers (12).Recent finding indicates that acute hyperglycemia in nondiabetic and Impaired glucose tolerance (IGT) subjects, elevate plasma IL-6 and tumor necrosis factor-α (TNF-α) concentration higher and longer in an individual with IGT and when glucose was given as pulse(13).By infusion of the antioxidant glutathione the effect was abolished, which suggests that hyperglycemia induced cytokine production is mediated by reactive oxygen species.

Subcutaneous and intraabdominal adipose tissue is a major source of TNF-α and IL-6 production (14).This raise the question of whether the acute phase reaction of type 2 diabetes is mainly secondary to obesity. In a recent study in which a case and control subjects were matched by body mass index (BMI) and waist circumference, neither CRP nor IL-6, predicted the development of type 2 diabetes, although lowered level of adiponectin did. Thus a hypothesis was suggested that because inflammatory markers are associated with obesity, they only indirectly predict diabetes and act as surrogate markers of hypoadeponectinemia (15).

Atherosclerosis is another co-segregate of type 2 diabetes which is strongly associated with acute phase response in its own right (16). Present evidence support the notions that atherosclerosis develops in parallel with type 2 diabetes (17) with both conditions showing the common antecedent of activated innate immunity, but like hyperglycemia and possibly some other manifestation of type 2 diabetes such as obesity, microangiopathy once present, would presumably further enhance inflammation.

Where there are studies on type 2 diabetes mellitus agreeing on the fact that acute phase proteins especially AAT are increased, studies in type 1 patients remains contradictory. It was found that acute phase markers are not elevated or may be mildly elevated in type 1 subjects who had the same degree and duration of hyperglycemia as type 2 subjects. At the same time, type 1 patients are at the same risk of developing atherosclerosis as the type 2 patient. Hence it was speculated that specifically diabetes related factors (possibly glucose) would need to be additionally sensitize the arteries to cytokines and other atherogenic factors such as hypercholesterolemia. It has also been shown that Type 1 diabetic patients have marginally higher plasma concentrations of AAT, suggesting the potential role of AAT in the pathogenesis of Type 1 diabetes. We have investigated the role of AAT as an inflammatory marker in pathogenesis of type 2 diabetes mellitus (18).

2. Materials and Method

2.1 Aims and objective

1.To detect the elevation of AAT , 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. 2. Compare the levels in newly diagnosed untreated type 2 diabetes mellitus patient with type 2 diabetes mellitus patients under treatment for at least 5 years.

3. Compare the level of this inflammatory marker of newly diagnosed untreated type 1 diabetes mellitus with newly diagnosed untreated type 2 diabetes mellitus patients.

2.2 Participants

Subjects were selected from various clinics and hospitals in Mangalore, India. 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. The study was approved by institutional ethical committee of Kasturba Medical College, Mangalore, India.

2.3 Materials

5 ml blood was collected in plain bottle. Informed consent was taken from the individual subjects prior to blood collection. Blood was taken from antecubital vein of the subjects and AAT assay in serum was carried out by the method of Sundaresh etal (19).

2.4 Principle of the test

The Proteolytic enzyme trypsin hydrolyses casein, with the formation of smaller peptides. The enzyme reaction after suitable interval of time is arrested by the addition of TCA which precipitated the protein, but the peptides are soluble in the acid. The TCA soluble fragments are a measure of Proteolytic activity of the enzyme. When the inhibitor is added to the preincubated mixture, it prevents the release of peptides by the Proteolytic enzymes. Thus the estimation of TCA soluble components in the presence and absence of inhibitor is a measure of inhibitory activity against Proteolytic enzymes.

The TCA soluble fragments are analyzed by the method of Lowry et al. The final color formed is a result of Biuret reaction of the peptides with copper ions in alkali and reduction of the phosphomolybdic acid reagent by the presence of tyrosine and Tryptophan which are present in the treated peptides.

2.5 Procedure

The mixture was preincubated at 37ºC for 10 minutes. The enzyme reaction was started by the addition of 1 ml of 2% casein. After 20 minutes incubation at 37ºC, the enzyme reaction was arrested by the addition of 3ml of 5% TCA. After standing for 30 minutes it was centrifuged for 15 minutes.

Contents	Enzyme blank	Enzyme control	Test blank	Test
Buffer	0.5 ml	0.5ml	0.5 ml	0.5 ml
Water	0.5ml	0.3ml	0.4 ml	0.2 ml
Diluted sample	-	-	0.1 ml	0.1 ml
Diluted enzyme 1: 300	-	0.2ml	-	0.2 ml

To 1 ml of supernatant, 1 ml of water and 4 ml of alkaline copper reagent were added. After 10 minutes, 0.4 ml of Folin reagents was added. Blue color developed was measured at 540 nm after 13 minutes.

The inhibitor concentration was calibrated in term of mgs of Trypsin inhibited, from the difference in the enzyme activity with and without sample. One unit of inhibitor activity is defined as 1 mg of enzyme inhibited under the assay condition.

2.6 Calculation

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

3. Results

The aim of the study was to examine the level of AAT as an inflammatory marker in pathogenesis in diabetes mellitus. The mean age (range), body mass index (BMI) and values of random blood sugar (RBS) are presented in table 1. The control group participants were so chosen as to cover the age range of the test groups. Table 2 lists the values of AAT in all four groups as mean ± SD. Table 3 denoted the comparison between different groups and significance levels (p values). In newly diagnosed type 2 diabetic patients (Group II) show higher level of AAT in compare to control group(Group IV) as depicts the significant p value(< 0.0001*). It's also detect that the level of the AAT is moderately higher in newly diagnosed type I(Group II) in compare to control (Group IV) as denoted by marginally significant p value (0.0002). It is also shown that level of AAT is even significantly higher (Group III) even after treatment by oral hypoglycemic drugs in compare to control group. (Group IV)

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

	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

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
n = umber of subjects SD = Standard Deviation
BMI= Body Mass Index
RBS= Random Blood Sugar

Table 2. The compare of mean value of AAT.

Serum Level (mg/dl)	Group I (Mean ± SD)	Group II (Mean ± SD)	Group III (Mean ± SD)	Group IV (Mean ± SD)
AAT (mg/dl)	495.70 ± 32.77	562.16 ± 63.00	519.38 ± 47.80	350.48 ± 114.07

Group I Group II Group III Group IV
SD = Standard Deviation
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

Table 3. Comparison of level of AAT (mg/dl) between different groups in Table-3 (p value < 0.05 is considered significant).

Comparison between groups	Level (mg/dl)	Level (mg/dl)	p value
Comparison between Group I and Group IV	495.70 ± 32.77 (I)	350.48 ± 114.07 (IV)	0.0002*
Comparison between Group II and Group IV	562.16 ± 63.00 (II)	350.48 ± 114.07 (IV)	< 0.0001*
Comparison between Group III and Group IV	519.38 ± 47.80 (III)	350.48 ± 114.07 (IV)	< 0.0001*
Comparison between Group I and Group II	495.70 ± 32.77 (I)	562.16 ± 63.00 (II)	0.003
Comparison between Group II and Group III	562.16 ± 63.00 (II)	519.38 ± 47.80 (III)	0.03

4. Discussion

The aim and objective of this study was to determine whether low grade chronic inflammation as a pathogenic cause in type 1 and type 2 diabetes mellitus or not. AAT levels were determined as a prominent inflammatory marker. In the twenty-five newly diagnosed type 2 patients the level of α1antitrypsin was found to be significantly increased as compared to control (Table 3).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 completely ruled out.

The underlying mechanism for the augmented acute phase response is not well understood and the stimulus for the response is unknown. Characteristic changes in liver protein synthesis occur in response to tissue damage from infection or injury known collectively as the acute phase response (20). In this setting, increased hepatic production of a group of socalled “acute phase reactant” proteins. AAT, together with creactive protein, ferritin, complement factors, and others are significantly up regulated by the result of IL-6-, TNFα-, and IL-1-driven signal transducer and activator of transcription 3 (STAT3) and nuclear factor kappa B (NF-κB) p65 signaling, while cortisol-binding globulin, transferrin, and albumin are decreased which are considered as negative acute phase proteins(21,22).

Circulating blood AAT levels in the acute phase have been found to increase three- to four fold (23). In addition to the acute phase response, AAT levels can also increased by other inflammatory settings including pregnancy, trauma, surgery, malignancy, and treatment with oral contraceptives(2428).Finally, there is some evidence that circulating granulocytes could contribute to local AAT levels through the transcription, storage, and release of AAT following migration into tissues(29-31). While some studies have demonstrated that AAT is stored in secretory vesicles within neutrophils (30,31), more recent data suggests that approximately80 % of neutrophil-associated AAT is localized to the cell membrane within lipid rafts, where it co- localizes with Fc fragment IgG receptor IIIb (FcγRIIIb) (29) .

Intriguingly, many of the anti-inflammatory charecters of AAT appear to be not dependent of its antiprotease activity. Churg and colleagues shown that oxidized AAT, lacking in antiproteolytic capacity, attenuated silica-induced increases in lung monocyte chemoattractant protein 1(MCP-1) expression, macrophage inflammatory protein- 2 α (MIP-2 α), activation of NF-κB, and associated neutrophilic migration into the lung (32-33). More recently, Jonigk and colleagues demonstrated that a recombinant form of AAT lacking elastase inhibitory function exhibited anti-inflammatory properties in lipopolysaccharide (LPS) challenged mice, decreased infiltration of neutrophils, decreased TNF-α, decreased lung tissue expression of DNA damage inducible transcript 3 protein (DDIT3) and X-box binding protein-1 (XBP-1) as well as in freshly isolated human blood neutrophil (decreased TNFα and IL-8 secretion ex vivo)(32-33).While antiprotease activity may not be necessary for AAT-mediated effects on inflammation, proper protein glycosylation does seem to be significant. Hyperglycemia and insulin resistance could promote inflammation and inflammation may be a factor linking diabetes mellitus to the development of atherosclerosis. Elevated glucose levels promote inflammation by increasing oxidative stress by increased formation of tumor necrosis factor (TNF) kappa B (34). In this study, the mean BMI was found to be 19.5 ± 1.23 in type 1 patient and 24.03 ± 1.46 in type 2 patients. No correlation was found between BMI and acute phase reactants. Hence it can be summarized that there could be multiple pathways involved in the activation of the innate immunity system and much work needed to be done to establish either a casual role in the development of mainly type 2 diabetes and could be type 1 diabetes also.

Having demonstrated that there is an inflammatory process going on in type 2 diabetes, we next thought of estimating inflammatory markers in patients on treatment (for at least 5 years) with oral hypoglycemic drugs. Many of the drugs have been shown to have anti-inflammatory effects. Statin drugs inhibits HMG-CoA reductase and prevent

atherosclerosis and inhibit the acute phase response by diminishing the deposition of low density lipoprotein (LDL) particles rich in cholesterol and phospholipids in macrophages and smooth muscle cells (35). Statins were found to reduce CRP levels and did not correlate with the reduction of the lipid levels suggesting that the in addition to their ability to reduce LDL, statins may also inhibit the acute phase response (36). Freeman DJ et al showed that statin therapy also prevent diabetes mellitus. Pravastatin therapy in the West of Scotland Coronary Prevention Study resulted in a 30% reduction of risk of developing type 2 diabetes(37). Salicylates in high doses have been known to lower glycosuria in diabetic patients (38). Although earlier studies were contradictory, these studies has used lower aspirin doses (<3gm/day) and therapeutic duration was only for a few days. Hundal RS (39) reported that high doses of aspirin (7 gm/day) for 2 wks caused 25% reduction in fasting plasma glucose, 50% reduction in triglyceride and 15 % reduction of CRP concentration independently of the changes in the plasma insulin concentration. Another widely used oral hypoglycemic agents thiazolidinedions (Glitazone) are peroxizome proliferators activated receptor γ (PRAR γ) agonist that have been regarded as insulin sensitizes through mechanisms such as altered transcription of insulin sensitive genes controlling lipogenesis, adipocytes differentiation, fatty acid uptake and GLUT 4 (Glucose Transporter 4) expression. They also have an anti-inflammatory action inhibiting cytokine production, macrophage activation and reducing CRP as well as WBC count in type 2 diabetic subjects (40-43).

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 (44) Torlone E et al demonstrated improved glycemic control in patients with arterial hypertension and type 2 diabetes using ACE inhibitors (45) Insulin has a potent anti-inflammatory activity. 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 (46).

In this study, of the 25 type 2 diabetic patients on treatment for at least 5 yrs, 8 patient were in sulfonylurea-metformin combination, 7 were on glitazone, 6 were on sulfonylurea alone, 2 were on glitazone-metformin combination and 2 were on metformin alone. In newly diagnosed untreated type 2 diabetic group (Group II) the levels of AAT is statistically elevated in compare to the control group (Group IV) which used to demonstrate that type 2 diabetes trigger the process of inflammation. When the level of AAT in treated group (Group III) compared with untreated group (Group II) not shown any statistically significance p value (0.03), that may be demonstrated that oral hypoglycemic therapy don't have any significant effect to reduce the inflammatory condition triggered by type 2 diabetic patients. Irrespective of oral hypoglycemic drug used for the treatment, treated group (Group III) showed significantly higher AAT levels comparable to the control group (Group IV).

5. Conclusion

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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