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RESEARCH ARTICLE

ASSOCIATION OF LOW GRADE CHRONIC INFLAMMATION AND ACTIVATED INNATE IMMUNE SYSTEM IN PATHOGENESIS OF NEWLY DIAGNOSED TYPE 2 DIABETIC PATIENTS AS WELL AS PATIENTS UNDER TREATMENT BY ORAL HYPOGLYCEMIC DRUG FOR MINIMUM 5 YRS

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ABSTRACT

Aim: Pathogenesis of type 2 diabetes is observed to be associated closely with acute phase response which is predominately cytokine-mediated. By estimating circulating acute phase proteins in type 2 (T-2) diabetic patients, I tried to establish this hypothesis.
Method: Newly diagnosed 25 T-2 cases and 25 T-2 cases under oral hypoglycemic agent for at least 5 years were chosen and were estimated the level of α1- antitrypsin, α1- acid glycoprotein, ceruloplasmin and fibrinogen. These all parameters are well known acute phase reactants and very important tools for diagnosis for low grade chronic inflammatory reaction. Thirty normal controls were also studied. The levels of these proteins were correlated with their random plasma glucose values and BMI.
Results: In the T-2 patients ($p<.00001$) in comparison with the controls, the values of all the four proteins studied were significantly elevated.
Conclusion: By the above results and findings is can be definitely postulated that a low grade inflammatory process is associated in the pathogenesis of type 2 diabetes. This can be further explored for diagnosis, management and follow up.

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INTRODUCTION

Diabetes Mellitus is one of the most common major public health problems having worldwide distribution. It has now adopted epidemic proportions. Overall it affects upto 30% of the world population and corners significant portion of total amounts spent on health care (Amos *et al*., 1997; Curry *et al*., 1997). In 1997-98 (Pickup and Crook, 1997) it was first proposed that in the pathogenesis of type 2 diabetes mellitus, chronic low grade inflammation and activation of the innate immune system are closely involved (Carmen, 2006). This can be considered as one of the recent explosion of interest in the notion. Several studies after that have shown that for the development of type 2 diabetes circulating markers of inflammation, acute phase reactants or interleukin-6 (IL-6) are strong predictors (Snijder *et al*, 2001; Spranger *et al*, 2003). α1- acid glycoprotein, α-1 antitrypsin, fibrinogen and ceruloplasmin are considered as few of the very important and accurate tools for measurement of low grade chronic in flammatory response leads to activated immune system. The level of these inflammatory markers in the pathogenesis of type 2 Diabetes mellitus was of interest in our present study. Type 2 diabetes mellitus is considered as a heterogeneous disorder with a complex etiology which used to develop in influences of environment and response to genetic. Central factor to the development of type 2 diabetes mellitus are insulin resistance and decreased secretion of insulin, although according to the maximum studies insulin resistance precedes insulin secretary defect (Defronzo, 1997). It has been more difficult to substantiate whether insulin deficiency rather than insulin resistance is the primary pathogenic mechanism in type 2 diabetes mellitus. The initial reports that insulin concentrations in patients of type 2 diabetes mellitus were comparable to healthy controls suggested impairment in insulin action rather than insulin secretion (Gerich, 1998). Recently, there in increasing evidence that for the development of type 2 diabetes mellitus and also associated complications such as dyslipidemia and atherosclerosis are closely associated with ongoing cytokine induced acute phase response which is sometimes called low grade inflammation, but part of aactivated innate immune system.High plasma levels of circulatory inflammatory markers such as C-reactive protein

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and interleukin-6 are predicted to be associated with the development of type 2 diabetes mellitus and both acute phase reactants and glycaemia are reduced by lot of drugs which have properties to fight against inflammation and possibly decrease the risk of developing type 2 diabetes mellitus.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. Levels of the individual acute phase proteins in plasma rise at different rates. First; the levels of C-reactive protein and α-1 antichymotrypsin rise, then within the first 12 hrs, α-1 acid glycoprotein followed by α-1 antitrypsin, haptoglobin, C4 and fibrinogen levels rise and finally there is a rise of C3 and ceruloplasmin levels. All levels reach their maximum within 2-5 days(Murray *et al*, 2002). Out of theall risk factors for development of type 2 diabetes mellitus factors like age, inactivity, certain dietary components, smoking, psychological stress and low birth weight are considered as highly associated with, that are also considered to be involved with activated innate immunity. For developing type 2 diabetes mellitus (Pickup andCrook, 1997) activated immunity may be consider as common antecedent. All of other associated signs and symptoms of type 2 diabetes mellitus such as fatigue, sleep disturbance and depression are likely to be at least partly due to hypercytokinemia and activated innate immunity. The body's rapid first line defense against environmental threat such as microbial infections, physical or chemical injuries is the natural or innate immune system. (Pickup,2004). A series of reactions are activated followed by that prevent ongoing tissue damage, isolate and demolished infective agents and activate repair mechanism to restore homeostasis (Medzhitov and Janeway, 2000). A major component of activated innate immunity is a series of sentinel cells such as macrophages, antigen presenting β cells and dendritic cells and probably also; intestinal epithelial cells, endothelium, kupffer cells in liver, adipocytes and others. These act as 'trouble detectors devices'. A number of germ line-encoded (i.e. monoclonal) pattern recognition receptors (PRPs) on and in these cells detect conserved molecular structures (pathogen associated molecular patterns) that are characteristic of a class of harmful agents. The most researched PRPs are probably the family of at least 10 tolls like receptors (TLRs) which are situated on the cell surface as transmembrane receptors (Fearon, 1997). Binding to PRP cause activation of nuclear factor Kappa β signaling pathways. This induces immune response genes, those for inflammatory cytokines which are considered as main mediators of low grade chronic inflammation and acute phase response (Medzhitou and Janeway, 2001). Acute phase reactant secretion from the liver is stimulated by cytokines. These also have positive impact on the brain to release adrenocorticotropic hormone and therefore secretion of cortisol from the adrenal gland leads to activation of sympathetic nervous systems leads the release of catecholamines. Acute phase response can be aggravated by psychological stressful situation via innervations of cells which produce cytokine and stimulation of the sympathetic nervous system and adrenergic receptors on macrophages. Recently it has been appreciated that a very prominent second function of innate immunity is to motivate the adaptive immune response (Jiang *et al*, 1998). Inflammation is a localized safeguard response to tissue injury. The word inflammation means 'setting of fire' and the process has been known from Egyptian times. The cardinal features of redness, swelling, heat and pain were described by Celsus (first century A.D) and loss of function was added by Galen (130-200AD) (Medzito and Janeway, 2002). Microscopically these features are due dilatation of capillaries, accumulation of leukocytes and augmented capillary permeability of intestinal fluid and stimulation of nerve endings by mediators such as substance P (Cone, 2001).

MATERIALS AND METHODS

Participants

Subjects were selected from various private clinics and Govt. hospitals in Mangalore, India. At first physicians examine the patient of previously undiagnosed diabetes and out of all confirmed cases, patients who suffered by chronic inflammatory diseases, history of recent acute inflammatory conditions, smokers, alcoholics, women consuming oral contraceptive pills or any other hormones, pregnant women, patients with clinical presentation of neuropathy, nephropathy, and retinopathy were not considered in the study. Twenty five (25) type 2 patients between the age limit of 30-60 yrs. of either sex participate in the study. Twenty five (25) type 2 diabetes mellitus patients who are under medication of oral hypoglycemic drugs for at least 5 yrs between the age limit of 30-60 yrs. were also chosen. Type 2 was decided based on age of patients and their subsequent response to insulin and oral hypoglycemic, respectively. Thirty (30) individuals were chosen from close relatives of the patients to serve as controls. The control groups were applied the same exclusion criteria like of test groups. Institutional Ethics Committee approved the total test protocol. Body mass index (BMI) was measured after recording of weight and height of each and every patients as well as controls. Random blood samples were collected before starting of the treatment in the diabetic patients.

Estimation of the following parameters was undertaken:
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 subjectsand followingestimations was carried out

Estimations

Estimation of Random plasma glucose (RBS): Using Hitachi 917 autoanalyser using Roche Kits I detect RBS by glucose oxidase method.

Fibrinogen estimation: In presence of calcium chloride fibrinogen in plasma was converted to fibrin. The fibrin clot was taken followed by digestion with NaOH. By using biuret method protein content of the clot was determined.(Varley, 1991)

Ceruloplasmin estimation: Paraphenylene diamine (PPD) is oxidized to get a coloured compound which is believed to correspond either to Bandrowski's base or to Weuster's red. This process is catalyzed by ceruloplasmin at pH 5.4. The rate of production of the coloured oxidized product is proportional to the concentration of ceruloplasmin, if a correction is done for the nonenzymatic oxidation of Paraphenylenediamine. Simultaneous determinations were carried out with and without sodium azide. Nonenzymatic oxidation of PPD used to inhibit by sodium azide. The ceruloplasmin concentration is proportional to difference between the values of the two tests.(Sunderman and Nomoto, 1970).

α-1 antitrypsin estimation: Proteolytic enzyme trypsin hydrolyze casein with the formation of smaller peptides. After suitable interval of time tri-chloroacetic acid (TCA) arrested the enzymatic reaction with the precipitation of the proteins. But the peptides are soluble in the acid. The TCA soluble fragments are a measure of proteolytic activity of this enzyme. When the inhibitor is mixed to the preincubated compound, it stops the release of peptides by the proteolytic enzymes. Thus, the measurement of TCA soluble compounds in the presence and absence of inhibitor is a estimation of inhibitory activity against proteolytic enzymes. The TCA soluble components were estimated by Lowry *et al* (Loway, 1951) method.The ultimatecolor developed is the end product of the reaction of the peptides with copper ions in alkali medium and reduction of the phosphomolybdic reagent by the presence of tyrosine and tryptophan which are component of the treated peptides. (Sundaresh *et al*, 1978).

Estimation of α-1 acid glycoprotein: By usingof perchloricacid heat coaguable proteins used to eliminate and the orosomucoid which remains present in the solution was precipitated by phosphotungstic acid and measured by determining its carbohydrate content by reaction with its tyrosine residues with folin ciocalteau reagent. (Winzler *et al*, 1955).

Statistics

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

RESULTS

By the entire study our aim was to determine whether low grade chronic inflammation with associated activated innate immune system is a pathogenic cause in freshly diagnosed type 2 diabetes mellitus cases or not.

Even we studied the association of this chronic inflammatory response as well as activated innate immune system in type 2 diabetic patients under therapy with oral hypoglycemic drugs.In table 1 the mean age (range), BMI and males/females ratio are shown. The control group participants were so taken as to cover the age range of the test groups.Lists the data of random blood sugar (RBS) and acute phase proteins in all three groups as mean ± SD are denoted in table 2. In comparison with the control group test group T- 2 had significant elevated result of all the parameters which can be further supported by analysis the significance levels (p values) of the T-2 versus control groups which are denoted in table 3. The level of ceruloplasmin and α1 acid glycoprotein were quite under control after treatment by oral hypoglycemic drug for 5 years, which can be further again depicts by the p values of the test and control groups.

DISCUSSION

This study was setup for detection whether pathogenicity of type 2 diabetes mellitus is related with low grade chronic inflammatory process with activated innate immunity or not.Increased levels of α1-antitrypsin, α1-acid glycoprotein, ceruloplasmin and fibrinogen were shown in twenty-five type 2 newly diagnosed patients. Our findings were in supporting with various authors who researched on acute phase proteins in type 2 diabetes (Defeo *et al*, 1993; McMillan, 1989; Festa *et al*, 2002). The importance of chronic low grade inflammation with activated innate immune system in the pathogenesis of type 2 diabetes seems possible out of doubt. The most dreaded and brutal complication being that of development of atherosclerosis resulting in cardiovascular diseases in which fibrinogen is determined as a sole risk factor in the production and manifestation of ischemic heart diseases. Ceruloplasmin is also an acute phase reactant with a response of intermediate magnitude and is known to have antioxidant action (Goldstein *et al*, 1979) and also known to stimulate cell proliferation and angiogenesis (Allessandri *et al*, 1983).

Table 1. Characteristics of subjects

Controls (n = 30)	Type 2 (n = 25)	Type2under treatment	(n=25)
Age	43.97±14.06 (30-60 yrs)	47.27 ±7.11 (30-60 yrs)	51.32 ± 7.56(30-60yrs)
BMI	20.75 ±2.27	23.03 ±1.46	24.20 ± 2.40
Males: Females	17 : 13	15 : 10	16:09

n= number of subjects

Table 2. Values of the acute phase proteins as Mean ±SD

Parameters	*Controls Mean ±SD*	*Type 2 Mean ±SD*	*Type2 under treatment Mean ±SD*
Random blood Sugar(mg/dL)	93.20±7.00	192.26±35.20	193.61 ± 33.65
α1 antitrypsin (mg/dL)	349.48±114.07	561.16±63.00	519.38 ± 47.80
α1 acid glycoprotein(mg/dL)	102.41±22.13	180.93±31.94	87.10 ± 17.69
Ceruloplasmin (mg/dL)	25.95±4.10	44.05±9.03	25.73 ± 9.94
Fibrinogen (mg/dL)	334.34±42.19	571.25±82.26	581.74 ± 79.09

Table 3. Comparison of p valuesbetween groups

Parameters	*T-2 v/s Controls*	*T-2UT v/s Controls*	*T-2 v/s T-2UT*
Random blood Sugar (mg/dL)	< 0.0001*	< 0.0001*	0.972
α1 antitrypsin(mg/dL)	< 0.0001*	< 0.0001*	0.03
α1 acid glycoprotein(mg/dL)	< 0.0001*	0.005	< 0.0001*
Ceruloplasmin(mg/dL)	< 0.0001*	0.55	< 0.0001*
Fibrinogen(mg/dL)	< 0.0001*	< 0.0001*	0.682

T-2= Type 2 newly diagnosed patient
T-2 UT = Type 2 diabetic patient under treatment for minimum 5 yrs
p ≤0.05 was considered as significant
**= statistically significant*

May be due to an oxidative stress that is prevalent intype2 diabetes (Telci *et al*, 2000; Baynes, 1991), the levels of ceruloplasmin in newly diagnosed type 2 are high as compared to controls. Eduardo Ehrenwaldshowed a very interesting characteristic of ceruloplasmin that the intact human ceruloplasmin which is 132 KD molecules produced elevated oxidation of LDL in vitro. Starkebaum G and Harlan JM *et al* also mentioned that excess oxidized LDL could be generated by increased serum concentration of ceruloplasmin and produce vascular injury by producing free radicals like hydrogen peroxide. The earlier notions of the antioxidant activity of ceruloplasmin can be defined and supported by these findings.

By further extensive investigations Eduardo Ehrenwald *et al* found that the holoceruloplasmin has a prooxidant effect and the action was attributed to the copper ions present in ceruloplasmin. These holoceruloplasmin is seen in serum as a 132 KD molecule. The commercially available ceruloplasmin which had an antioxidant effect, is mainly a degraded product containing either 115 KD fragment or 19 KD fragment or both. The works done to show that ceruloplasmin used these degraded products as an antioxidant. Even in the system the antioxidant action of a commercial ceruloplasmin was observed where holoceruloplasmin oxidized LDL (Ehrenwald *et al*, 1994). Hence it'squite debatable for considering ceruloplasmin as an antioxidant in vivo. The LDL oxidizing action of ceruloplasmin could probably explain at least in part of the increased risk of IHD in type 2 diabetes. Also it could not be wrong to count ceruloplasmin as an acute phase reactant and important parameter whose levels used to increasein case of low grade chronic inflammatory condition as well as in activated innate immunity as seen in type2 diabetes. The values of various parameters when we compare between controls and type 2 patients reveal a significant elevated in type 2 patients (Table-2). Even the ceruloplasmin concentration was slightly higher in the type 2 patients, although it's not statistically very significant. The mean random blood sugar (RBS) values in Type 2 newly diagnosed diabetics was 192.26 ± 35.20 mg/dl.

The inflammatory markers levels were elevated in the type 2 patients. In spite of this huge difference, 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 before the clinical manifestation of hyperglycemia appear, (Engstrom *et al*, 2003; Schmidt *et al*, 1999; Duncan *et al*, 1999; Pradhan *et al*, 2001) inflammatory markers used to elevate well. This also gives credibility and support to the thought that activation of innate immunity is not related to hyperglycemia. But research has shown that the concentration of acute phase reactants (Gavella *et al*, 2003) used to decrease by decreasing plasma glucose levels.Also the inflammatory markers showed positive correlation with 2 hrs post load glucose values in few studies (Sriharan *et al*, 2002). Although number of hypotheses has been put forward but the underlying procedure for the augmented acute phase response is not well studied and the stimulus for the response is not known. Some of few hypotheses included resistance of insulin, obesity, atherosclerosis, other complicationsrelated to diabetes and maladaptation of the normal innate immune response to environmental threats (Pickup and Crooke, 1998; Grimble, 2002; Pradhan and Ridkar, 2002). The most widely studied is the association of obesity, insulin resistance type 2 diabetes and acute phase reactants. It has been studiedthat in the postprandial state (Mohammed *et al*, 1997; Hotamisligil *et al*,1998; Fried *et al*, 1998) adipocytes secret a number of proinflammatory cytokine. The term 'diabecity' has received attention (Duncan *et al*, 2003) of late for obese diabetics. The involvement of inflammation in the pathogenesis of diabetes and atherosclerosis is proposed and evaluated by 'common soil' theory. Inflammation can be promoted by hyperglycemia and insulin resistance and inflammation may be a factor linking diabetes mellitus to the development of atherosclerosis.Increased levels of glucose stimulated inflammatory reaction by increasing oxidative stress (Bayens and Thorpe, 1999) by the formation of AGEs and increased Tumor Necrosis Factor (kappa B) (Brownlee, 2001).

In our present study, the mean BMI was found to be 20.75 ± 2.27 in control and 23.03 ± 1.46 in new type 2 diabetic patients. No association 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 production of mainly type 2 diabetes. Having demonstrated that low grade inflammatory process is involved with the pathogenesis of 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 drugshave anti-in flammatory effects. Statin drugs inhibits HMG-CoA reductase and prevent atherosclerosis and inhibit the acute phase response (Bayens andThorpe, 1999). Statins though found to reduce CRP levels but 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 (Sparrow *et al*, 2001).Freeman DJ *et al* showed that statin medication also prevent diabetes mellitus. 30% reduction of risk of developing type 2 diabetes (Freeman *et al*, 2001) was resulted by Pravastatin therapy which was shown in the West of Scotland Coronary Prevention Study. Salicylates in high doses have been known to lower glycosuria in diabetic patients (Yuan *et al*, 2001).

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 (Hundal *et al*, 2002) reported that 25% decrease in fasting plasma glucose, 50% decrease in triglyceride and 15 % decrease of CRP concentration independently of the changes in the plasma insulin concentration are caused by high doses of aspirin (7 gm/day) for 2 weeks duration of treatment.Thiazolidinedione (Glitazone)is a widely used peroxisome proliferators activated receptor γ (PRAR γ) agonist agents 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 (Ricole *et al*, 1998; Haffner *et al*, 2002; Chu *et al*, 2002; Ebeling *et al*, 1999). In type 1 as well as in type 2 diabetic patients, Angiotensin Converting Enzyme Inhibitors (ACE inhibitors) are also known to decrease insulin resistance with concomitant hypertension (Pollare *et al*, 1989). Torlone E *et al* demonstrated that using ACE inhibitors improved glycemic control in patients with arterial hypertension and type 2 diabetic.

(Torlone *et al*, 1993) Insulin has a potent anti-inflammatory activity which 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 specific acute phase gene (Campus and Baumann, 1992). In our present 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. The levels of α1-antitrypsin, α1-acid glycoprotein and ceruloplasmin were statistically lower when compared with newly diagnosed untreated group.

The levels of fibrinogen did not show any significant difference. The values of α1-acid glycoprotein and ceruloplasmin were comparable to those of the control group. The values of RBS were similar to those of untreated group (193.61 ± 33.65 and 192.26 ± 35.30). It is very interesting phenomena to notice that α1-acid glycoprotein levels were quite low in under treatment patients in compare to type 2 diabetic and even control populations. This is probably because of α1-acid glycoprotein is the most amenable acute phase protein to treatment modalities. Comparable ceruloplasmin levels in type 2 patients on treatment and controls again raise the question as to the 'prooxidant' or 'antioxidant' action of ceruloplasmin. No change in fibrinogen values suggest multiple pathway involvement that are poorly understands and need further studies to come to a definite conclusion.

Conclusion

For decades 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. With this, the accepted pathogenesis also becomes questionable. 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 of activated innate immunity in the development 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 in a small subset of population. 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.

Conflict of interest: None

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