SERUM REGENERATING ISLET-DERIVED 1 ALPHA (REG1α) PROTEIN LEVELS AS BIOMARKER FOR TYPE 1 & 2 DIABETICS

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ABSTRACT

OBJECTIVES: 1) To compare serum levels of regenerating islet-derived 1 alpha (Reg1α) proteins in type 1 diabetics, type 2 diabetics and controls and correlate them with their clinical/biochemical parameters.

2) To compare Reg1α levels in diabetics with and without disease complications.

METHODS: In this cross sectional comparative study, ten type 1 diabetics and thirty type 2 diabetics were recruited from Medical Department of PNS Shifa Hospital, Karachi. Twenty controls were selected from among friends and relatives with non diabetic background. Biochemical parameters like Fasting Blood Glucose (FBG), Glycosylated Hemoglobin (HbA1c), Total Cholesterol (TC) and Triglycerides (TG) were measured. Serum levels of Reg1α were analyzed using ELISA.

RESULTS: Reg1α protein levels, compared to controls were significantly higher (p <001) in both type 1 and 2 diabetics with increase more discernible in case of type 2. A decrease in the Reg1α levels was observed with increase in the duration of the disease in type 2 diabetes patients. However the levels of the protein remained significantly higher than the controls. Patients with diabetic complications had higher protein levels as compared to diabetics without complications. We observed positive correlation of Reg1α with age at onset of disease in case of type 1 diabetics (p<0.05). Patients with risk factors like high body mass index and smoking had higher protein levels.

CONCLUSION: Reg1α proteins in types 1 and 2 diabetes patients can be used as biological marker for detection of β-cell apoptosis and regeneration independently. It may be used to identify patients with disease complications.

KEY WORDS: Diabetes Mellitus (MeSH); Hemoglobin A, Glycosylated (MeSH); Blood HbA1c protein, human (MeSH); Regenerating Islet-Derived 1 alpha Protein (MeSH), Diabetes complications (MeSH).

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INTRODUCTION

Pancreatic β-cell deficiency leading to hyperglycemia is a key feature of both type 1 and type 2 diabetes. Type 1 diabetes is characterized by autoimmune destruction of insulin producing pancreatic β-cells leading to all-time dependency on exogenous insulin.[1] Insulin resistance, hyperinsulinemia and β-cell apoptosis are the characteristic features of type 2 diabetes. Some of the mechanisms that have been proposed to induce β-cell apoptosis in type 2 diabetes includes glucolipotoxicity, islet amyloid polypeptide (IAPP), oxidative stress, endoplasmic reticulum stress and inflammatory cytokines.[2] Autopsy studies are the only available means for detection of β-cell death and they report 70% to 100% deficiency in β-cell mass in case of type 1 diabetes and upto 65% in type 2 diabetes.[3] Human β-cells have restricted aptitude for regeneration. Widespread studies going on in the search for the growth factors involved in the regeneration of β cells and cure of diabetes lead to the discovery of Regenerating islet derived (Reg) family proteins.

The probable roles for Reg gene in replication, growth, and maturation of insulin producing cells was suggested, when expression of this gene was found in regenerating and hyperplastic islets but not in normal islets.[4] Reg1 protein, the normal product of acinar cells, has a growth promoting effect on β-cells and improved experimental diabetes.[5-7] Reg protein acts on pancreatic β-cells in either/both autocrine or paracrine mode and a receptor that convey the growth signal of Reg protein for rejuvenation of β-cells have been identified.[8] Islet cells isolated from Reg knockout mice showed a decreased 3(H) thymidine incorporation while β-cell specific over expression of Reg gene showed increased proliferation in isolated islet cells and showed a delay in the onset of diabetes,[9] indicating the role of Reg1 gene in β-cell growth and regeneration. Reduction of acinar product Reg1 was associated with the pathological process involved in impaired glucose tolerance of pancreatitis-linked

diabetes and increasing age,[10] while Reg1 protein administration improved the insulin secretory capability of islet β cells, indicating its role in the therapeutics of diabetes. Reg gene expression have been found to be up regulated in experimental models of type 1 and type 2 diabetes involving production of cytokines particularly interleukin-6 (IL-6),[11,12] and a response element for IL-6 has been identified in Reg1 gene promoter region.[13]

Reg proteins are acute phase proteins which belong to the C-type lectins super family. In human five members of the Reg family protein are known and Reg1α is the first member to have been identified and is encoded by an approximately 2.7 kb gene located on chromosome 2p12.11 Reg protein expression was found to be increased in the islets from a diabetic patient, and anti-Reg antibodies were detected in the NOD mouse[14] and in some diabetic patients.[15] Reg1α protein has been found to be expressed in ductal cells, acinar cells, and in regenerating islet β-cells of pancreas in human and its expression is upregulated in type 1 and 2 diabetes in an endeavor to regenerate β-cells destroyed either due to autoimmunity or glucolipotoxicity. There is considerable difference between the pathogenesis of the two types of diabetes. Keeping this in mind the aim of this study was to characterize the circulatory Reg1α levels in both type 1 and type 2 diabetics and associate them with the clinical/biochemical parameters and with disease associated complications.

METHODS

Subjects

We studied 40 diabetic subjects of different disease duration from the Medical Department of PNS Shifa Hospital and 20 euglycemic subjects with non diabetic background in this cross sectional comparative study (June 2014 to November 2014). Blood samples of study subjects were collected and stored for further analysis. Reg1α protein were measured in the serum of the following patient groups: 10 type 1 diabetics with different age groups and disease duration, 30 type 2 diabetics and 20 normal subjects sex / age matched. Both type 1 and type 2 diabetics were evaluated according to the ADA criteria.[16]

Among the selected diabetics, patients with known diabetic complications were also included in the study. Macro vascular complications included Ischemic heart disease (IHD) and diabetic foot. While micro vascular complication included diabetic nephropathy, retinopathy, and neuropathy. Diabetic patients with cataract and skin manifestations like carbuncle were also included in the study. Diabetic patients with IHD were diagnosed on the basis of clinical findings and investigations like EGG, angiography and thallium scan. Diabetic foot and neuropathy were diagnosed on the basis of clinical examination, electromyography (EMG) and nerve conduction studies (NCS). Patients with nephropathy had more than double the normal levels of 24 hours urinary protein, decreased creatinine clearance and deranged renal function tests (RFTs). For diagnosis of diabetic retinopathy, clinical examination and fundoscopy was done. Cataract was diagnosed on the basis of ophthalmological examination by slit lamp. Patient with carbuncle was diagnosed on the basis of dermatological examination. The study does not include patients with other chronic diseases like hepatitis B and C, inflammatory bowl diseases and cancers. The Ethical Committee of Army Medical College approved the study protocol and written approval for serum analysis was acquired from each subject. Each study subject was examined in the morning after an overnight fast. Height, weight, systolic and diastolic blood pressure were measured. Blood samples for measurement of Reg1α and other biochemical parameters (FBS, HbA1c, TC and TG) were taken.

Reg1α levels in human

The serum Reg1α levels were measured using a human Reg1α Bioassay ELISA Kit (US Biological, life Science), which is a very sensitive sandwich type of enzyme immunoassay for quantitative assessment of Reg1α in plasma, serum and other biological fluids in humans. Serum samples were diluted twice with 0.01M Phosphate Buffer Saline (PBS) to bring the final concentration to 1:500. Equal amount of blank, standards and samples (100ul) were added to wells of ELISA plate coated with human Reg1α specific antibody in duplicates. The micro titer ELISA plate was incubated for 2 hrs at room temperature. Hundred micro liter of the Reagent A working solution (Biotin-conjugated antibody specific to Reg1α) was added to each well and incubated for 1 hr at 37°C. After first washing 100ul of the Reagent B working solution (Avidin-conjugated with HRP) was added to each well and incubated for 30 min at 37°C. After second washing incubation with 90ul TMB substrate was done for 15 min at 37°C. Reading was taken fifteen to twenty minutes after adding Stop Solution, at 450 nm wavelength. A standard curve between absorbance values versus Reg1α concentrations of standards was obtained. The results are interpreted as the Reg1α concentration (pg/ml) in samples. The actual quantity of Reg1α in the serum sample was calculated by multiplying the test result by 500 (dilution factor). Readings were converted to ng/ml after multiplying by 0.001. The antibodies used in this ELISA had no known cross reactivities with any recombinant human Reg1β, PAP, and Reg IV protein as they were specific for human Reg1α.

Statistical Analysis:

Statistical analysis was carried out using version 16 of SPSS statistical software. Descriptive statistics were calculated. Shapiro Wilk test were applied to check the normality of the data. Mean ± SD OR Median (Inter Quartile Range) were calculated. The Mann-Whitney U-test and the Kruskal-Wallis test were used to compare quantitative variables. Spearman's rank correlation test was used to investigate correlations between variables. P-values of less than 0.05 were considered significant.

RESULTS

Clinical and demographic characters of the study groups

The clinical and demographic characters of the study groups are shown in Table 1. Compared with the control subjects, type 2 diabetics had raised mean BMI, raised systolic and diastolic blood pressure and raised fasting blood glucose (FBG), HbA1c, total cholesterol (TC) and triglyceride (TG) levels. Type 1 diabetics also had higher levels of FBG and HbA1c as compared to controls.

Descriptive analysis of patients with diabetic complications is shown in Table II. From among 40 diabetics, 14 (35%) were reported to have one or more disease complications. These included three patients with type1 diabetes and eleven patients with type 2 diabetes.

Serum Reg1α levels in type 1 and type 2 diabetics

Raised level of protein was observed in type 1 and type 2 diabetics compared to controls ($p < 0.001$). Raised Reg1α protein levels were also observed in type 1 diabetics compared to normal subjects ($p = 001$). Type 2 diabetics also demonstrated raised protein levels compared to normal subjects ($p < 001$). Reg1α protein levels in type 2 diabetics were higher as compared to the Reg1α protein levels in type 1 diabetics although the difference was not statistically significant (Figure 1).

In type 2 diabetics, correlation between time span of the disease and protein levels showed a decrease of serum Reg1α protein levels in years. ($p = 0.06$; Spearman $r = -0.348$).

Serum Reg1α level correlates positively with age at disease onset in type 1 diabetics

In case of type 1 diabetics lower serum levels of the protein were observed with age at the onset of the disease less than 26 years as compared to diabetics with age more than 26 years at disease onset ($p<0.05$) (Figure 2).

TABLE 1: CLINICAL AND DEMOGRAPHIC CHARACTERS OF THE STUDY GROUPS

Parameters	Control (n=20)	Type I (n=10)	Type II (n=30)	P value
Sex, M/F	11/9	7/3	20/10	—
Age, years	51±3	34±16	56±7	—
Age at onset of disease, years	—	24.2±14.2	48.4±7.6	—
Duration of Disease, years	—	9.8±6.04	8.2±6.2	—
Body Mass Index, kg/m2	24±4	22.3±5.2	25.2±3.3	NS
Systolic Blood Pressure, mmHg	120(110-120)	120(115-125)	130(120-140)	<0.001*
Diastolic Blood Pressure, mmHg	80(70-80)	80(76-82)	80(80-90)	<0.001*
Fasting Blood Glucose, mmol/L	3.7(3.5-4.4)	10.6(7.4-17)	7.2(6.0-9.1)	<0.001*
HbA1c, %	5.5(5.4-5.6)	10.3(8.8-12)	7.6(6.9-9.1)	<0.001*
Total cholesterol mmol/L	3.7(3.2-4.7)	3.6(3.5-4.6)	5.1(4.5-5.6)	<0.001*
Triglyceride TG, mmol/L	1.2(0.9-1.2)	1.5(0.9-1.7)	1.7(1.2-2.3)	=0.079

TABLE II: DESCRIPTIVE ANALYSIS OF DIABETES COMPLICATIONS

Diabetes Complications	Patients With Complications (n=14/40)		
	Frequency (n)	Percent (%)	Valid Percent (%)
Ishemic Heart Disease	4	10	28.6
Cataract	3	7.5	21.4
Diabetic foot	1	2.5	7.1
Carbuncle	1	2.5	7.1
Neuropathy	1	2.5	7.1
Nephropathy	1	2.5	7.1
IHD/Diabetic foot	1	2.5	7.1
Neuropathy/Retinopathy	2	5	14.3
Total	14	35	100

Serum Reg1α level are higher in patient with diabetic complications

Serum levels of Reg1α were measured in controls, diabetics without complications and diabetics with complications. A significant difference was found between the three groups with $p<0.001$ (Mean Rank control = 15.26, diabetics without complications = 30.90 and diabetics with complications = 48.32) Serum Reg1α levels were significantly raised in diabetics without complications as compared to normal subjects ($p=0.001$), diabetic with complications as compared to controls $p<0.001$) and diabetics with complications as compared to diabetics without complications ($p<0.001$) (Figure 3).

Serum Reg1α level and Metabolic Factors

In case of type 2 diabetics' positive correlation between Reg1α and BSF, HbA1c was found but it was not statisti-

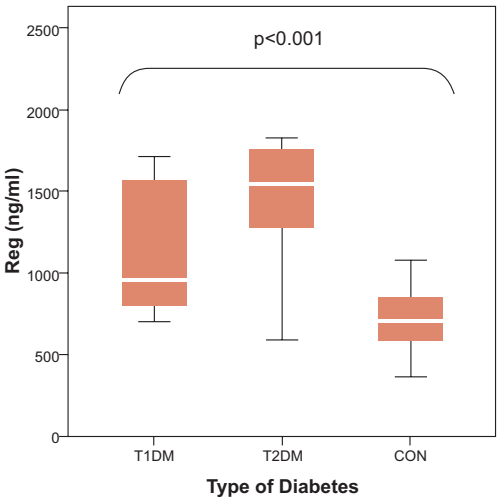

Figure 1: Serum Reg1α levels in controls, type 1 diabetics and type 2 diabetics

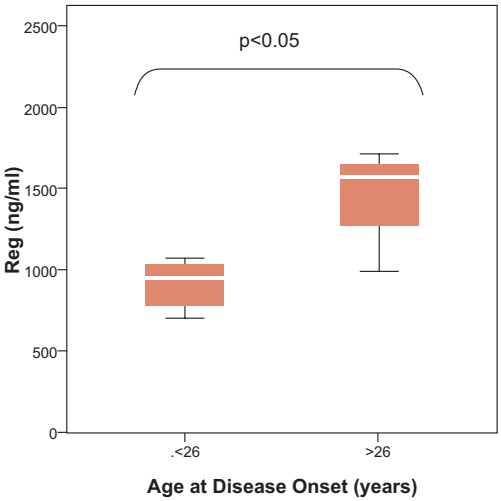

Figure 2: Serum levels of Reg1α in type 1 diabetics depending on age at disease onset

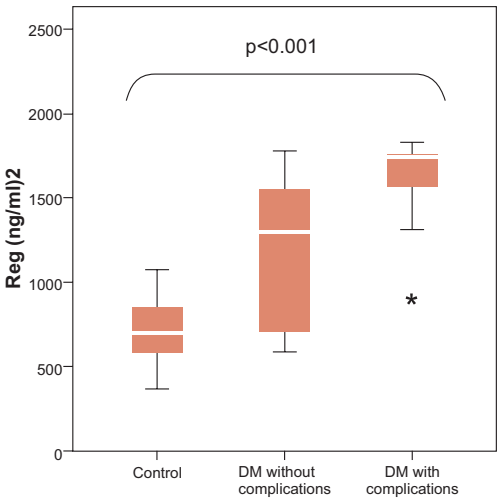

Figure 3: Serum levels of Reg1α in controls, diabetics without disease complications and with disease complications

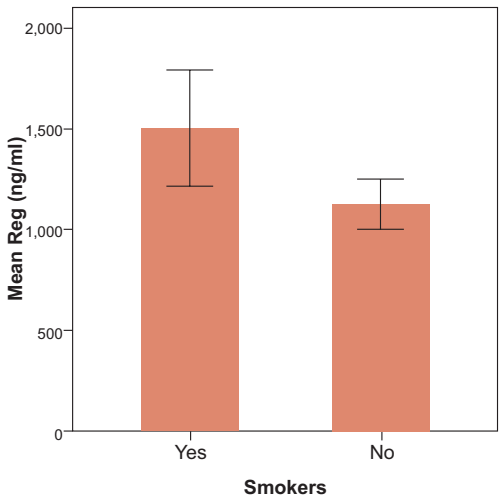

Figure 4: Comparison of serum levels of Reg1α in diabetics with and without history of smoking

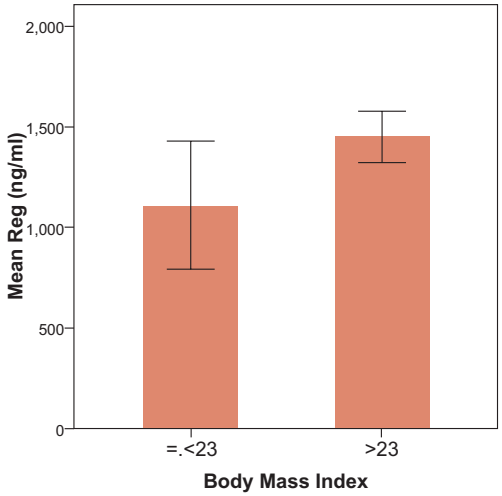

Figure 5: BMI and serum Reg1α levels in diabetics

cally significant. However a negative though insignificant correlation was seen in case of type 1 diabetics. No correlation was seen between protein levels and TC and TG in both type 1 and type 2 diabetics

Serum Reg1α level and the risk factors

Smokers had higher levels of the protein as compared to the non smokers but the data was not statistically significant (p=0.09) (Figure 4). Patients with increase BMI had raised levels of the protein with p=0.12 (Figure 5).

Family history of diabetes did not show any correlation with the Reg1α serum levels in both type 1 and type 2 diabetics.

DISCUSSION

Deficit of β-cell mass plays a major role in the pathogenesis of diabetes. Reg1 is a secretory protein which acts as a growth factor for these insulin producing cells. In the present study serum Reg1α levels in both type 1 and type 2 diabetic patients were found sig-

nificantly raised compared to normal subjects. Amongst the diabetics, the levels were more elevated in type 2 diabetics. Raised levels of Reg1α have been reported previously in both type 1 and type 2 diabetics and also in the patients with other forms of diabetes.[17-19] However no such work has been reported in our part of the world, where every tenth person is suffering from this life threatening and debilitating disease. In type 1 diabetes, the experimental models have shown an increased expression of Reg1 genes after loss of α-cells, due to local penetration of immune cells.[14] The role of inflammation in type 2 diabetes has also been reported by many studies.[20,21] In islets of rat models of type 2 diabetes, increased expression of Reg1 was observed in association with peri-islet immune cell infiltration and release of cytokines.[12] Treatment of β-cells by IL-6 and Dexamethasone (Dx) together induced Reg1β expression.[22] It was demonstrated that apoptotic β-cells stimulate Reg gene expression in neighboring cells, to facilitate β-cell regeneration thereby linking β-cell apoptosis and their regeneration.[23] Hyperglycemia[23] and inflammation along with β-cell damage in both type 1 and type 2 diabetes leads to increase expression of Reg1α gene in both the regenerating islets and in exocrine pancreas.

Although type 1 diabetics belong to all age groups, the major contributors are children and young adults. In present study, among the ten subjects with type 1 diabetes half of them had relatively low protein levels irrespective of the duration of the disease. These patients were diagnosed with diabetes at a very young age, two of them were siblings. These patients had relatively higher FBG and HbA1c levels and were totally dependent on insulin and did not respond to oral hypoglycemic agents. The reason could be the complete β-cell loss at the clinical onset of the disease, or these may be the cases with auto-antibodies to the Reg1α, as antibodies against Reg1α has been demonstrated in approximately 50% of the type 1 diabetes patients[15,17] indicating more aggressive nature of the disease. These patients also had a strong family history of the disease. Patients with disease onset in adulthood had higher levels of the Reg1α and relatively lower FBG and HbA1c levels indicating less aggressive nature of the disease or phenotype suggestive of regeneration of β-cell in these cases. No correlation between disease time span and serum protein levels was found in case of type 1 diabetes in our study.

The type 2 diabetes patients were much older. They showed decrease in the level of the Reg1α protein with the increase in their age, though, no significant correlation was found between age at the onset of the disease and serum Reg1α levels. A negative correlation between time span and serum Reg1α levels was observed in type 2 diabetics. With the increase in the duration of the disease the metabolic demand of the cells increases while the regeneration capacity of β-cells decreases in humans because with the increase in age the ability of β-cells to replicate decreases.[24] In type 2 diabetes with the increase in the duration of disease a reduction in functional β-cell mass occurs.[25] A decline in the Reg1 expression has been observed during the aging process and age-related islet β-cells dysfunction.[26] In pancreatitis-linked diabetes the decline in acinar cells, the major source of Reg1 has been demonstrated.[10] Thus, the relative decrease in Reg1 levels with age and disease time span may be due to substantial damage to β-cells that occur in late stages of type 2 diabetes, decrease in their regeneration capacity and a decrease in acinar cells. Patients with high levels of Reg1α had higher levels of FBG and HbA1c compared to the patients with low Reg1α levels in case of type 2 diabetics. It has been reported that high glucose level potentiates Reg gene expression.[23,27]

In this study raised levels of Reg1α were demonstrated in diabetic patients with complications irrespective of the duration of disease. In developing countries like Pakistan most of the patients with diabetes are diagnosed late and may present for the first time with diabetes complications. A recent study by Yang and colleagues, related increased levels of Reg1 with the clinical stages of the disease and its associated complications.[19] Reg1α is the product of acinar cells under normal conditions, however under pathological conditions other sources of the protein like regenerating islets, stomach and intestine etc. have been reported. Increased Reg1 levels are related to the severity of inflammation and may serve as a biomarker for post-trauma complications.[28] Recent studies have reported raised post-operative serum Reg1 levels in patients of cardiac surgery with infection[29] and in patients with sepsis.[30] More studies are needed to locate the exact source of raised Reg1α in diabetes patients with complications.

The patients with autoimmune type 1 diabetes had lower BMI compared to type 2 diabetes patients which is associated with obesity related insulin resistance. In this study, patients with low BMI had lower levels of the protein. This may be due to the fact that mass of β-cell is less in lean compared to the obese subjects.[31] Obesity itself is associated with low grade inflammation when linked with diabetes;[32] it may lead to increased expression of Reg1α, as is seen in obese mice models where inflammatory markers were elevated.[12] Moreover, obesity related insulin resistance and hyperglycemia[23] may also lead to increase in Reg1α levels. Higher protein levels in smokers in this study is possibly due to nicotine in cigarettes, which has been linked with apoptosis of β-cells.[33] A recent study has shown raised levels of Reg1α in smokers with inflammatory lung disease like chronic obstructive pulmonary disease.[34]

CONCLUSION

Reg1α proteins in both type 1 and type 2 diabetes patients may be used as biological marker for detection of β-cell

apoptosis and regeneration independent of other variables. A diverse expression pattern of Reg1α was observed in both types of diabetes especially when compared with the clinical and biochemical parameters. These differences may be due to the difference in the pathogenesis of the two types of diabetes. Reg1α may be used to identify patients with disease complications. However animal and human studies with larger sample size are required to verify these results, especially in our part of the world where environmental factors, different lifestyles, diverse dietary habits and genetics may have a profound effect on the expression of this protein.

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AUTHOR'S CONTRIBUTION

Following authors have made substantial contributions to the manuscript as under:

SSU: Study design, acquisition analysis and interpretation of data, drafting the manuscript, final approval of the version to be published.

AKN, SB, HA: Concept & study design, final approval of the version to be published. Critical revision, drafting the manuscript, final approval of the version to be published.

Authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

CONFLICT OF INTEREST

Authors declare no conflict of interest

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