ORIGINAL RESEARCH

Serum levels of p53 and cytochrome c in subjects with type 2 diabetes and impaired glucose tolerance

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Abstract

Purpose: To examine apoptotic markers in serum of subjects with diabetes and impaired glucose tolerance (IGT). Serum levels of p53 and cytochrome c, regulator molecules for apoptosis, were measured in subjects with type 2 diabetes, subjects with IGT and healthy controls.

Methods: Forty one subjects with type 2 diabetes, 27 with IGT and 27 healthy volunteers were included in the study. Serum level of cytochrome c and p53 were measured with competitive ELISA.

Results: Serum levels of p53 were lower in the group of subjects with type 2 diabetes (0.85 ± 0.39 U/ml) than in controls (1.09 ± 0.49 U/ml) ($P < 0.05$) and in the subjects with IGT (0.98 ± 0.37 U/ml) ($P < 0.05$). There was no significant difference between the group with IGT and controls. Also, there was no difference for serum level of cytochrome c among the groups. In the group of subjects with type 2 diabetes, serum level of cytochrome c was mildly correlated with HbA1c ($r: 0.39$, $P < 0.05$).

Conclusion: The present study shows that the serum level of p53 is lower in the patients with type 2 diabetes than in controls or in subjects with IGT. No difference was seen among the the groups for the serum level of cytochrome c.

The ability to secrete adequate amounts of insulin is determined by the functional integrity of β -cells and

their overall mass.¹ There is increasing evidence that β -cell apoptosis and impaired proliferation consequent to hyperglycaemia is one pathway which could be responsible for initiation and progression of diabetes.¹⁻⁴ Apoptosis of β -cells in type 2 diabetes is caused not only by glucotoxicity but also by lipotoxicity, amyloid accumulation in islet cells, oxidative stress and endoplasmic reticulum stress.⁵⁻⁷ Apoptosis of β -cells mainly occurs via death receptors (Fas). Elevated glucose concentrations induce apoptosis in human β -cells due to an interaction between constitutively expressed Fas ligand and upregulated Fas.³ As a result of interaction between apoptotic signals and cell-surface receptors, cytochrome c is released from mitochondria by the mediation of some cytosolic proteins.^{8,9} In the cytoplasm, the complex formed by cytochrome c and caspases (special proteases) leads activation of other caspases. Finally, activated caspases activate the endonucleases which are responsible for degradation of DNA.¹⁰ Cytochrome c released from apoptotic cells is an important marker of apopto-

sis and its serum level has been determined in various studies.¹¹⁻¹³

p53 is considered to be a stress response gene. p53 protein acts to induce cell cycle arrest or apoptosis in response to DNA damage. In the case of cell cycle arrest, the cell gains enough time to repair damage and, thereby, genetic stability is maintained in the organism.¹⁴ Hyperglycaemia induces apoptosis by p53 and activation of cytochrome c-activated caspase-3 pathway.¹⁵

On the basis of those data we hypothesized that circulating levels of cytochrome c and p53 may have prognostic value for type 2 diabetes, and/or may be reliable markers for IGT. In this case, blood concentrations of molecules regulating apoptosis may be useful in early diagnosis of type 2 diabetes. Therapeutic approaches designed to arrest apoptosis could be an important new development in the management of type 2 diabetes. No previous study has examined the serum concentrations of cytochrome c and p53 in patients with diabetes.

Materials and Methods

The study received the approval of the Cerrahpasa Medical Faculty Ethics Committee in accordance with the principles of Declaration of Helsinki and informed consent was obtained from all subjects.

Forty one subjects with type 2 diabetes (18 men and 23 women) and 27 subjects with IGT (11 men and 16 women) were recruited from the Endocrinology, Metabolism and Diabetes Department of Cerrahpasa Medical Faculty, Istanbul University. Subjects with type 2 diabetes had been diagnosed based on common clinical and laboratory findings. Eight of the subjects were smokers (1cigarette/day). None was taking alcohol for at least two years. The mean duration of the disease was 8 ± 2 yr. Thirty two of the subjects were being treated with anti-diabetic agents (21 subjects were taking metformin, and 11 subjects were taking metformin+gliclazide). Twenty eight of the diabetic subjects had hyperlipidemia and were taking statins.

Thirty one of the diabetic subjects had hypertension and were taking ACE inhibitors. Fourteen subjects with type 2 diabetes presented with at least one complication such as retinopathy, neuropathy, nephropathy or angiopathy. Other subjects were free from diabetic complications. Subjects with fasting glucose between 110-126 mg/dl and glucose concentration between 140-200 mg/dl at the second hour of Oral Glucose Tolerance test constituted the IGT group. Three subjects were smokers (1cigarette/day) and none was taking alcohol for at least two years. They were not receiving medication and were not supplemented by antioxidant vitamins. They had normal liver, thyroid and renal function. Subjects with acute and/or chronic inflammatory disease, autoimmune disease, thyroid disorder and cancer, subjects who were taking alcohol and any medication, and subjects who smoke more than 1cigaret/day were excluded from the study. The control group was constituted by age-matched 27 healthy individuals (12 men and 15 women) who recruited among staff of the Cerrahpasa Medical Faculty. None of the controls had a family history of diabetes and they had normal glucose tolerance. None of controls were smokers or were taking alcohol for two years as in the other groups. None of the controls received vitamins or any drugs. Characteristics of type 2 diabetes, IGT and control groups are shown in table 1.

TABLE1. Characteristics of the study groups

	Type 2DM n=41	IGT n=27	Control n=27
Mean age (year)	55 $\pm$ 10	49 $\pm$ 10	50 $\pm$ 9*
BMI (kg/m ²)	28 $\pm$ 4	29 $\pm$ 3	28 $\pm$ 4
Fasting glucose (mg/dl)	114 $\pm$ 20	101 $\pm$ 9**	89 $\pm$ 7†
Serum C-peptide (ng/ml)	3.27 $\pm$ 1.27	3.63 $\pm$ 1.82	3.87 $\pm$ 1.03*
HbA _{1c} (%)	6.4 $\pm$ 0.7	6.0 $\pm$ 0.5††	5.3 $\pm$ 0.4†

*P<0.05 vs type 2 DM,

**P<0.005 vs type 2 DM

†P<0.001 vs type 2 DM and IGT

††P<0.01 vs type 2 DM

Blood sampling and measurement of cytochrome c and p53

Venous blood samples, 5 ml, were collected after an overnight fast. After centrifugation at 3000 X g for 10 min, serum was removed and kept at the -80°C until analysis. Serum levels of cytochrome c and p53 were measured with competitive ELISA kits obtained from Chemicon International, Temecula, CA, USA, and Bender MedSystems, Burlingame, CA, USA, respectively. The ELISAs for the p53 and cytochrome c were performed per the manufacturers instructions.

Serum levels of glucose and c-peptide were measured, in the routine analysis laboratory, by spectrophotometric and chemiluminescent methods, respectively. HbA1c levels in erythrocytes were determined by HPLC in the same laboratory.

Statistical analysis

Measured parameters are expressed as mean $\pm$ SD. Data were analysed by one-way ANOVA. The diabetic group was divided into subgroups with respect to glycemic control (subjects with HbA1c >6.5 were considered poorly controlled diabetics, and subjects with HbA1c <6.5 were considered well controlled) and with respect to the presence of diabetic complications; and comparisons between groups were made by Mann-Whitney U test. Differences among groups were considered significant at $P<0.05$. Spearman correlation coefficient was used for correlation analysis.

Results

Serum levels of p53 were lower in subjects with type 2 diabetes than in both controls ($P<0.05$) and subjects with IGT ($P<0.05$). No difference was found between the group of subjects with IGT and controls for serum level of p53 (table 2). There was no significant difference among the groups for cytochrome c. No differences were found between non-smokers and smokers for parameters either in subjects with diabetes or in subjects with IGT. When the diabetic group was di-

vided into subgroups with respect to glycemic control and diabetic complications, no differences were found between well and poorly controlled diabetics for either cytochrome c or p53. No differences were found between subjects with or without diabetic complications (table 2). In subjects with type 2 diabetes, the serum level of cytochrome c was weakly correlated with HbA1c ($r:0.39$, $P<0.05$). No further correlations were determined between apoptotic markers and duration of diabetes and clinical indicators of diabetes such as serum level of glucose and c-peptide. Serum level of p53 was weakly correlated with BMI in the subjects with IGT ($r:0.41$, $P<0.05$).

Discussion

The serum level of p53, a protein which has a central role in apoptosis, is lower in subjects with type 2 diabetes than in both healthy controls and subjects with IGT. There is increasing evidence that apoptosis plays a pivotal role in the decrease of beta-cell mass in diabetes.¹⁶⁻¹⁸ Chronically elevated glucose has been suggested as a major responsible factor for increased apoptosis in pancreatic β -cells (16). Kim et al.¹⁶ have shown that chronic exposure to high glucose increases apoptotic cell death, which is mediated by cytochrome c release and caspase-3 activation, in a mouse pancreatic beta-cell line. Recent studies in pancreatic β -cell lines, knockout mice, human and rat islets revealed that apoptosis is the main mode of pan-

TABLE 2. Apoptotic parameters in the study groups

	p53 (U/ml)	Cytochrome c (ng/ml)
Type 2 DM	0.85 $\pm$ 0.39*	0.97 $\pm$ 0.22
Poorly controlled diabetics (n=15)	0.82 $\pm$ 0.48	0.95 $\pm$ 0.29
Well controlled diabetics (n=26)	1.08 $\pm$ 0.28	
Patients with diabetic complications (n=14)	0.83 $\pm$ 0.43	0.95 $\pm$ 0.29
Patients without diabetic complications (n=27)	0.89 $\pm$ 0.36	0.98 $\pm$ 0.20
IGT	0.98 $\pm$ 0.37	0.96 $\pm$ 0.19
Control	1.09 $\pm$ 0.49	1.17 $\pm$ 0.39

* $P<0.05$ versus IGT and Control groups

creatic β -cell death, not only in type I but also in type II diabetes.¹⁹

Circulating levels of cytochrome c have been determined in only a few studies, by Ben-Ari et al.¹² in patients with liver disease, by Adachi et al.²⁰ in patients with systemic inflammatory response syndrome and by Barczyk et al.²¹ in cancer patients receiving therapy. No previous study has addressed the serum level of cytochrome c in diabetic subjects. At the beginning of the present study we had expected greater serum levels of cytochrome c in subjects with type 2 diabetes and IGT than in controls. Surprisingly, there was no difference among the groups for serum level of cytochrome c. This may have been due to utilization of cytochrome c in the cytosol, by binding to cytosolic proteins, to induce caspase-3 activation. Thus, it was not elevated in serum.

Zheng et al.²² reported that p53-deficient mice were more susceptible to streptozotocin-induced diabetes than control mice, suggesting that p53 may have a protective role in pancreatic β -cell death. In an established mouse model for diabetes in which the MHC class I antigen is overexpressed in pancreatic beta cells, beta cell death is elevated compared to wild-type mice. However, there was no increase in immuno-reactivity towards anti-p53 antibodies in the pancreas of these transgenic mice over the course of diabetes formation and beta cell death.²³ These data suggest that the classical cell death pathway dependent on p53 may not be operating in pancreatic beta cells. There is little previous data for serum levels of p53. Kolomecki et al.²⁴ and Malviya et al.²⁵ measured p53 levels in patients with primary follicular thyroid tumours and urinary bladder tumours. In general, baseline p53 level determined in the serum may be derived from cell turnover. In the case of induced apoptosis, serum p53 level may increase. We were unable to find any study examining serum level of p53 in diabetics. Higher p53 levels in subjects with type 2 diabetes than in the controls would have been attributed to apoptotic death of pancreatic β -cells. However, serum p53 was lower in type 2 diabetics. This

may imply that the classical cell death pathway dependent on p53 may not be operating in pancreatic beta cells, as suggested by Nam et al.²³ Alternatively, hormonal, immunological and biochemical alterations in diabetes, synthesis of p53 may be suppressed. On the other hand, the choice of p53 as well as cytochrome c as apoptotic markers may not be appropriate. Circulating Fas ligand and M30 antigen, which is a caspase-degraded product of cytokeratin, may be more reliable markers for apoptosis.

In conclusion, it has been determined that the serum level of p53 is lower in patients with type 2 diabetes than in controls or in a group of subjects with IGT. No difference was found between the three study groups for cytochrome c. This is a preliminary study with a limited number of parameters and a limited number of subjects. We propose to continue our investigations and examine different apoptotic markers in the circulation of larger groups of diabetic subjects.

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