CLINICAL STUDY

Serum reg protein level is not related to the beta cell destruction/regeneration process during early phases of diabetogenesis in type I diabetes

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Abstract

Objective: In type I diabetes mellitus, early markers of beta cell damage are needed in order to detect the infraclinical development of the disease. The reg protein may be a good candidate, as the reg gene has been proposed to play a role in the pancreatic beta cell destruction/regeneration process during diabetogenesis in animal models of autoimmune diabetes. The aim of this study was to test the hypothesis whether serum reg protein level could be representative of either the destructive or regenerative process at the beta cell level during the early phases of type I diabetes in humans.

Design and methods: We used a highly specific immunoassay to measure serum reg protein level in controls and in three groups of either diabetes prone or diabetic subjects: recently diagnosed diabetic patients, long-standing diabetic patients and islet cell antibody-positive non-diabetic subjects.

Results: We found no significant difference between the values observed in these three groups in comparison with control group (90.7 ± 18.1 ng/ml, 83.1 ± 5.6 ng/ml, 98.7 ± 24.5 ng/ml vs 85.5 ± 5.6 ng/ml respectively). Moreover, when the insulin reserve was evaluated at 6 months in the recently diagnosed group, serum reg protein levels were not different between patients with or without residual insulin secretion (at onset: 103 ± 42 vs 70.3 ± 8.5 ng/ml respectively; at 6 months: 79.7 ± 25.8 ng/ml vs 81.6 ± 15 ng/ml respectively). In contrast, trypsin levels were significantly lower in every group of diabetic patients. Results were expressed as means $\pm$ S.E.M. and groups compared by Student's *t*-test ($P < 0.05$).

Conclusions: We conclude that serum reg protein level cannot be used as a marker for the progression of the diabetogenic process in type I diabetes.

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Introduction

Type I diabetes mellitus is an autoimmune disease characterized by the selective destruction of the insulin-secreting pancreatic beta cells. The clinical onset is preceded by a silent period of variable duration (pre-type I diabetes) during which the autoimmune reaction progresses. Screening for pre-type I is a challenge that would allow an early therapeutic intervention, before the overt diabetes. Such diagnosis is at the present time based on a combination of immunological (islet-cell autoantibodies as ICA, GAD-A and IA2-A and anti-insulin antibodies) and metabolic (acute insulin response during i.v. glucose tolerance test) markers (1–3). However, these markers lack specificity. Indeed, chronic anti beta cell autoimmunity has been described without development of diabetes (1, 4). Moreover, alteration of the first phase insulin response to glucose

represents a late marker of beta cell damage (5–7). A successful therapeutic intervention, occurring as soon as possible in the natural history of type I diabetes, should require the use of marker(s) reflecting the progression of the diabetogenic process. Indeed, measurement of reg protein might be a good candidate for this purpose. The reg protein is normally secreted by exocrine pancreatic cells and reg gene overexpression has previously been shown to be associated with regeneration of pancreatic islets (8–11). In animal models of autoimmune diabetes, pancreatic overexpression of reg mRNA has been reported (12–14) during active phases of diabetogenesis and reg gene proposed to play a role in the destruction/regeneration process during autoimmune aggression of beta cells (reviewed in ref. 15). In humans, as in rodents, there are a number of arguments which indicate that regenerative phenomena may occur during diabetogenesis. Gepts (16) has

observed some beta cell enriched islets in the pancreas of diabetic patients who died a few months after clinical onset of the disease, suggesting that some regenerative processes occur during this phase of the disease. Transient glucose intolerances associated with autoimmune markers have been noted in twin studies, confirming that, on rare occasions, beta cells could overcome autoimmune aggression (4). Regeneration of endocrine cells from the duct, mainly with the glucagon cell phenotype, has been also noted by O'Reilly (17) in the non-obese diabetic (NOD) mouse. As exocrine pancreatic proteins can be measured in blood, we made the assumption that serum reg protein levels might reflect pancreatic expression during the early phases of the disease in diabetic patients, and then these could be a marker of the destruction/regeneration balance. Accordingly, the reg protein level was measured in either recently diagnosed diabetic patients or first relatives who were at risk of diabetes, due to exhibiting positive high titer islet cell antibodies. These levels were compared with both a control population and a group of long-standing diabetic patients. In addition, reg protein levels were correlated to the presence or absence of detectable C-peptide secretion during the first year of the disease in order that it might reflect the possible regenerative process during the honeymoon period of the disease.

Material and methods

Subjects

Seventy-two subjects were retrospectively studied:

- (1) Twenty-two adult controls (blood donors from the Centre Régional de Transfusion Sanguine, Marseille, aged 20–50 years) without any diabetic relative and not suffering from diabetes, pancreatic or digestive diseases.
- (2) Twenty-one recently diagnosed diabetic patients (14 males and 7 females aged 24–46 years). The patients were treated at onset by intensive insulin treatment with continuous insulin infusion during 5–7 days, then followed by conventional insulin therapy with 2–3 daily subcutaneous injections. Blood samples had been taken at onset and at 6 and 12 months following diagnosis. In parallel, beta-cell reserve was evaluated by measuring plasma C-peptide before and after i.v. injection of 1 mg glucagon (Glucagen, Novo-Nordisk, Boulogne-Billancourt, France) using a commercially available kit. This test was performed only if fasting plasma glucose was in the 4.4–11 mmol/l range.
- (3) Twenty long-standing diabetic patients (15 males and 5 females aged 4–46 years, duration of diabetes 2–35 years) with no residual insulin secretion (e.g. undetectable plasma C-peptide at fasting).
- (4) Nine non-diabetic subjects (five males and four females, aged 6–40 years, first degree relatives of type I diabetic patients) with high titers of islet cell antibodies (ICA) (>40 JDF units). This group was considered as being at high risk of type I diabetes. Indeed, seven out of

these nine subjects later became diabetic (assays were performed on blood samples taken 1–5 years before clinical onset). One is still not diabetic. One was lost.

Immunoassays

Preparation of serum samples Blood samples collected in standard tubes were taken in the morning after an overnight fast, allowed to clot at room temperature, and centrifuged for 10 min at 1700 g. The sera were then stored frozen at –20 °C until assay.

Immunoenzymatic assay of the reg protein Serum reg protein levels were measured by direct sandwich immunoassay as previously described (18) on 0.1 ml serum diluted to a final volume of 0.3 ml in the diluting medium (0.2 mol/l phosphate buffer, pH 7.2, 10% rabbit serum). As a solid phase, 6.5 mm diameter polystyrene balls were coated with specific IgG, and horseradish peroxidase labeled IgG was used as a secondary antibody. Peroxidase activity was measured using *o*-phenylenediamine dihydrochloride as substrate. The sensitivity of the assay was 0.3 ng/ml and the within-run and day-to-day coefficients of variation around 10% consistent with most ELISA procedures. As previously published (19), it was checked that antibodies prepared against reg protein isolated from pancreatic juice did not react with another member of the reg superfamily such as PAP/HIP protein.

Determination of serum trypsinogen concentration

Blood trypsinogen levels were measured by an immuno-enzymatic assay as previously described (20). The assay was shown to specifically recognize the major human trypsinogen:trypsinogen 1 or 'cationic' trypsinogen.

ICAs

ICAs were measured by indirect immunofluorescence on cryostat sections of human pancreas (blood group O) in accordance with Juvenile Diabetes Foundation (JDF) standardization workshops (threshold limit in our laboratory > 5 JDF units).

Statistical analysis

Results were expressed in mean $\pm$ S.E.M. They were compared using a two-tailed Student *t*-test and the significance threshold was $P < 0.05$.

Results

Serum reg values in recently diagnosed and in pre-type I diabetes are not different to that found in controls and long-standing diabetes

In recently diagnosed patients, mean serum reg protein levels were 90.7 ± 18.1 ng/ml at onset, and 77.5 ± 8.4 ng/ml and 67.5 ± 4.0 ng/ml at 6 and 12 months following diagnosis, respectively. Although

mean serum reg protein levels tended to decrease, no significant difference was observed during the 1-year follow-up. These values were not significantly different from the control values (85.5 ± 5.6 ng/ml). Two isolated high values (440 and 220 ng/ml) were noted in two different patients at onset and 6 months following diagnosis respectively, without evidence of clinical or biological particularities in these patients.

In long-standing diabetic patients, mean serum reg protein level was 83.1 ± 4.8 ng/ml. Two values higher than 1000 ng/ml were not taken into account because they concerned patients with terminal chronic renal failure. No correlation was observed between serum reg protein level and duration of diabetes.

In ICA-positive subjects, serum reg protein levels were 98.6 ± 24.5 ng/ml. These values were not significantly different from control values. We observed an isolated high value of 292 ng/ml in a patient whose evolution to diabetes is not known. One patient out of the nine studied is still not diabetic: serum reg protein level in this patient did not differ from that in the others (80 ng/ml). Distribution of individual serum reg protein levels in recently diagnosed diabetic patients, long-standing diabetic patients and ICA positive subjects is shown in Fig. 1.

Lack of relationship between serum reg protein levels and residual beta cell function during the first year of the disease

We compared two subgroups of the recently diagnosed diabetic population which differed only in the quality of

the residual insulin reserve during the 12 months following clinical onset. After 6 months, 9 out of 19 patients presented a marked residual insulin secretion (defined as a plasma C-peptide level higher than 0.5 nmol/l, 5 min after an intravenous infusion of glucagon). Serum reg protein levels in these nine patients did not differ from the remaining ten who were considered to have no or low insulin reserve (103 ± 42 ng/ml and 79.7 ± 25.8 ng/ml vs 70.3 ± 8.5 ng/ml and 81.6 ± 15 ng/ml at onset and 6 months following diagnosis, respectively) (Fig. 2). No correlation was observed between serum reg protein values and post-glucagon C-peptide levels at any time during the first year follow-up of the disease.

Serum trypsin levels were decreased in diabetic patients

The fasting serum values of trypsin in recently diagnosed and long-lasting diabetic patients were significantly decreased in comparison with controls (14.8 ± 1.24 ng/ml and 11.3 ± 1.3 ng/ml vs 23.28 ± 1.58 ng/ml respectively, $P < 0.001$). In contrast, serum trypsin levels of ICA-positive first degree relatives were not statistically different from the controls (16.7 ± 2.63 ng/ml). In the diabetic subjects there was a tendency to a progressive decrease of trypsin levels with the duration of the disease, but the differences reached the significant threshold only when one-tailed test was used; at diagnosis: 14.8 ± 5.69 ng/ml, 6 months later:

Figure 1 Distribution of serum reg protein levels in controls, patients with either recently diagnosed (M0: clinical onset, M6 and M12: 6 and 12 months later, respectively) or long-standing type I diabetes and subjects considered to be at risk of diabetes (ICA positivity, seven became diabetic later), represented on a semi-logarithmic scale.

Figure 2 Serum reg protein levels in recently diagnosed type I diabetic patients at 6 (M6) and 12 (M12) months as a function of the presence of residual insulin secretion (●, patients with a post-glucagon C-peptide value higher than 0.5 nmol/l; ○, values below this limit at 6 months).

14.3 ± 1.13 ng/ml, 12 months later: 13 ± 1.11 ng/ml, long-standing diabetics: 11 ± 1.3 ng/ml. No correlation was found between reg protein and trypsin levels in any group or the whole population.

Discussion

This pilot study shows that the measurement of serum reg protein levels with a highly sensitive and specific method failed to detect the destruction/regeneration phenomenon in the pancreas of type I diabetic patients. Indeed, in both ICA-positive pre-diabetic subjects and recently diagnosed diabetic patients, serum reg protein levels were not different to that found in the control group. In addition, during the year following diagnosis of clinical diabetes, the course of serum reg protein levels was similar in two groups differing only in the quality of the residual insulin reserve. Therefore, in our study, plasma reg protein level was influenced neither by the diabetogenic risk before the disease, nor by the residual beta cell mass during the honeymoon period. The high variability of serum reg protein levels between individuals is also worth noting, and has previously been reported by others (21, 22). Nevertheless, pancreatic overexpression of reg gene has been observed in murine autoimmune diabetes such as BB rats and NOD mice. In these diabetic prone strains, expression of reg gene was specially increased during the active phase of diabetogenesis, e.g. 90-day-old diabetes prone BB rats (12) or NOD female mice and cyclophosphamide-injected NOD male mice (13, 14).

Unfortunately, the serum reg protein level does not seem to reliably reflect the pancreatic production, at

least not in human type I diabetes. This discrepancy between secretion and plasma level has already been observed for some other proteins produced by exocrine pancreatic cells. For instance, lactoferrin, which is secreted in excess into pancreatic juice during chronic calcifying pancreatitis, has been found at a normal level in blood of patients suffering from this disease (23). In contrast to reg protein, some changes of serum trypsin values have been observed in diabetic patients. There was a decrease of this parameter, which had the tendency to be more pronounced with a longer duration of the disease. These results are in agreement with previously published data (24, 25). The lower trypsin levels in plasma of insulin-dependent diabetic patients have been attributed to a decrease of exocrine pancreatic secretion in this disease (26, 27). Even if a ratio reg protein/trypsin was considered in this study, the highest values were observed mainly in long-standing diabetes, and not in the more active periods of diabetogenesis. The lack of absolute or relative increase of reg protein in serum during early phases of type I diabetes can be explained by several reasons. Indeed, in animal models of islet regeneration, reg seems to act mainly as an autocrine/paracrine factor. Islet regeneration could not be transferred by parabiotic experiments in a model of cellophane-wrapping of the pancreas in the hamster (28). In another model (ligation of pancreatic duct), it was shown that no regeneration occurs downstream the ligation (29). Very similar results were reported in a third model of islet neogenesis by selective alloxan infusion in mice (30). Moreover, it is noteworthy that serum reg immunoreactive material can have extra-pancreatic sources including small bowels, gastric

mucosa or kidney as reg gene expression has been identified in all three tissues (31). We had the opportunity to study a patient with a subtotal pancreatectomy (results not shown). The serum reg protein level was within the normal range, despite undetectable serum immunoreactive trypsin. This result is in agreement with findings from our group and others of an absence of correlation between reg protein and immunoreactive trypsin serum levels in 160 subjects including controls and patients with cystic fibrosis or chronic pancreatitis (19, 22). We had the opportunity to measure reg protein levels in serum of controls during nutritional and pharmacological explorations; 36-h fasting (61 ± 19 to 64 ± 11 ng/ml, $n=2$), oral glucose load (fasting: 80 ± 17 , 60 min post load: 82 ± 18 , 120 min post load: 76 ± 10 ng/ml, $n=3$), fat-enriched test meal (77 ± 10 to 77 ± 11 ng/ml, $n=2$) and insulin-induced hypoglycemia (68 ± 19 to 70 ± 26 ng/ml, $n=2$) (unpublished results). Any of these tests was able to induce a significant rise of serum reg protein values. Another possible explanation for the discrepancies between human and animal data could be the fact that reg gene overexpression could be a very transient phenomenon. In various animal models of islet regeneration, reg gene overexpression occurs very early in pancreas (1–2 days) and lasts only a few days (less than 1 week) (8–11). In our series, it is noteworthy that three patients in both prediabetic and diabetic groups exhibited transient high serum reg levels. None of these patients suffered from acute or chronic pancreatitis, cancer of the digestive tract or chronic renal failure, pathologies in which elevated reg serum levels have been previously reported (21, 22). Transient high production from the pancreatic site cannot be excluded in these subjects.

In conclusion, these results are somewhat disappointing, as a reliable serum marker of beta cell damage is needed in order to improve the diagnostic value of the usual immune markers of pretype I diabetes (auto-antibodies as ICA, GAD-A, IA2-A and insulin auto-antibodies) and precisely predict the speed of the beta cell destruction. The beta cell function abnormalities such as the low acute insulin response to i.v. glucose seem to be too late a marker, occurring just 1–2 years before clinical onset. Despite the absence of predictive value of serum reg protein level in the at-risk patients, we can hope that improvement of the knowledge of endocrine/exocrine interactions in the pancreas submitted to autoimmune aggression will clarify the role of this protein during diabetogenesis in the future.

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