INCREASED LEVELS OF SOLUBLE FAS IN SERUM FROM DIABETIC PATIENTS WITH NEUROPATHY

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SUMMARY - Objective: The aim of this study was to investigate circulating soluble Fas (sFas) and Fas ligand (sFasL), two transmembrane glycoproteins involved in apoptosis, in the serum of diabetic patients.

Material and methods: We assessed sFas and sFasL serum levels in normal controls (n = 15), and in both 42 diabetic patients without complications, or with predominant retinopathy or neuropathy, using sFas and sFasL specific ELISA method.

Results: sFasL serum levels were less than 0.1 ng/ml in normal controls and in each group of diabetic patients. In diabetic patients with a predominant neuropathy, sFas serum levels were significantly increased not only when compared with normal controls (13.5 ± 3.6 ng/ml vs 7.1 ± 1.1 ng/ml, p < 0.001), but also when compared with patients without complications (vs 9.1 ± 1.8 ng/ml, p < 0.001) or with a predominant retinopathy (vs 8.7 ± 1.9 ng/ml, p < 0.001).

Conclusions: These preliminary data suggest that a dysregulation of the Fas system in peripheral neuronal cells may be involved in the increase of sFas observed in diabetic patients with neuropathy.

Key-words: neuropathy, apoptosis, diabetes, sFas, sFasL.

RÉSUMÉ - Augmentation des taux sériques de Fas soluble chez les diabétiques présentant une neuropathie.

Objectif : Le but de ce travail était de doser, dans le sérum des diabétiques, les formes solubles de Fas (sFas) et de Fas ligand (sFasL), deux glycoprotéines transmembranaires intervenant dans l’apoptose.

Matériel et méthodes : Nous avons dosé sFas et sFasL par ELISA dans le sérum de témoins (n = 15) et de 42 patients diabétiques, sans complications, ou ayant comme complication majeure une rétinopathie, ou une neuropathie.

Résultats : Les taux sériques de sFasL ont été trouvés inférieurs à 0,1 ng/ml chez les témoins et chez tous les patients diabétiques. Par contre, chez les patients diabétiques avec une neuropathie prédominante, les taux sériques de sFas étaient augmentés de façon significative non seulement par rapport aux témoins (13,5 ± 3,6 ng/ml vs 7,1 ± 1,1 ng/ml, p < 0,001), mais aussi par rapport aux patients diabétiques sans complications (vs 9,1 ± 1,8 ng/ml, p < 0,001) ou avec une rétinopathie prédominante (vs 8,7 ± 1,9 ng/ml, p < 0,001).

Conclusions : Ces résultats préliminaires suggèrent que, chez les patients diabétiques ayant une neuropathie prédominante, il pourrait exister une dysrégulation du système apoptotique Fas-dépendant au niveau du système neuronal périphérique.

Mots-clés : neuropathie, apoptosis, diabète, sFas, sFasL.
Apoptosis, or programmed cell death, is a physiological process observed in many organs and cells, which may also occur in pathological conditions. Several mechanisms of apoptosis have been described [1]. One of the most investigated pathways, the Fas system, is composed of Fas Ligand (FasL), a type II transmembrane glycoprotein and Fas antigen (Fas/Apo-1/CD95), a type I transmembrane glycoprotein receptor [2]. Cross-linking of Fas by FasL triggers apoptosis in various target cells [3].

Soluble isoforms of FasL (sFasL) and Fas (sFas) have been detected in human serum. Increased serum levels of sFasL have been reported in patients with autoimmune and neurodegenerative diseases, viral infections or malignancies [4]. Elevated serum levels of sFas have been found in patients with cardiovascular diseases and various autoimmune thyroid diseases [5-6]. Recent studies indicated that a dysregulation in Fas-mediated apoptosis was involved in both the insulitis process and the pancreatic β cell death. Indeed, in the pancreas of newly diagnosed type 1 diabetic patients, the Langerhans islets are infiltrated by FasL expressing T lymphocytes while the few remaining β cells are strongly Fas positive [7]. More recently, pancreas biopsy specimens of patients with recent-onset type 1 diabetes were analysed and it was observed that in inflamed islets β cells expressed Fas, and islet-infiltrating mononuclear cells were Fas L positive [8].

In type 2 diabetes, characterized by the association of relative insulin deficiency and insulin resistance, apoptotic mechanisms of apoptosis have been described [9]. One of the most investigated pathways, the Fas system, is composed of Fas Ligand (FasL), a type II transmembrane glycoprotein and Fas antigen (Fas/Apo-1/CD95), a type I transmembrane glycoprotein receptor [2]. Cross-linking of Fas by FasL triggers apoptosis in various target cells [3].

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In type 2 diabetes, characterized by the association of relative insulin deficiency and insulin resistance, apoptosis is now suggested in some experimental models and human implications have been evoked [9].

On the other hand, previous reports have suggested that a proapoptotic factor(s) may exist in sera from diabetic patients and play a role in the pathogenesis of diabetic neuropathy. Pittinger et al. have shown that sera from type 1 diabetic patients with neuropathy had cytotoxic effects on a N1E115 murine neuroblastoma (NB) cell line [10]. They observed that Fas was expressed on these NB cells, suggesting that the serum from these patients contained a Fas-mediated neuronal apoptosis activator. Srinivasan et al. have observed that the serum from type 2 diabetic patients with neuropathy induced apoptosis in a human neuroblas-

Assessment of diabetic complications

Diabetic retinopathy was assessed by direct ophtalmoscopy through dilated pupils and with fluorescein angiography. If a patient had five or more microaneurysms per eye in the fluorescein angiogram, a mild or non proliferative retinopathy was considered to be present. It was evaluated as proliferative in the presence of retinal neovessels [12]. Diagnosis of peripheral diabetic neuropathy was established from:

1) A detailed clinical symptom questionnaire including: paraesthesias, cutaneous hyperaesthesias, burning pain, lancinating pain, numbness, tingling, cramps in legs or feet.

2) A clinical examination, considered as essential [13], including: superficial sensitivity assessed by the Semmes-Weinstein 10 g standard microfilament, pin-prick, temperature sensation; conveying vibratory sensation (tested with a tuning-fork) and proprioception; muscle and tendon reflex testing: ankle, knee and tendon jerks.

3) Routine electrophysiological studies: sensory nerve conduction determined along the sural, cubital and median nerves; motor nerve conduction velocity, distal and proximal latencies determined along the sciatic, cubital and median nerves; in addition, electromyography was performed in two to five distal and proximal muscles in the lower and upper limbs. Patients were considered as having neuropathy in the presence of abnormalities in the clinical examination and of two or more alterations in electrophysiological evaluations.

**Abbreviations**

- DN: diabetic patients with a predominant neuropathy
- DR: diabetic patients with a predominant retinopathy
- DW: diabetic patients without complications
- EC: endothelial cells
- NB: N1E115 murine neuroblastoma
- NC: normal controls
- PMN: peripheral mononuclear blood cells
- sFas: soluble Fas
- sFasL: soluble Fas ligand.
Serum collection

Three groups of diabetic patients were established according to diabetic complications:
− DW (n = 18): diabetic patients without diabetic complications — excepted one with incipiens nephropathy (urinary albumin excretion > 30 mg/24 h. and < 300 mg/24 h.). Nine patients in this group were insulin-treated during their hospitalisation.

Plasma creatinine and urinary albumin excretion levels obtained at the end of hospitalisation after equilibration of diabetes are given Tables II, III and IV.
− DR (n = 10): diabetic patients with a predominant retinopathy. Mild or nonproliferative retinopathy was present in six patients, proliferative retinopathy in two, and severe proliferative retinopathy in one. One patient suffered from optic nerve ischemia. Incipiens nephropathy was detected in three patients. Seven patients in this group were insulin-treated.
− DN (n = 14): diabetic patients with a predominant neuropathy. Two patients were hospitalized for diabetic foot complications (one with ulceration and the other for amputation) and another for a severe cruralgia. Axonal sensory-motor polyneuropathy was predominant to the lower limbs in five patients, to the upper limbs in one, and present to the four limbs in five patients. Incipiens nephropathy was observed in two patients. Seven patients were insulin-treated.
− NC (n = 15): normal controls. This group consisted in normal subjects without any family history of diabetes and normal fasting plasma glucose.

Blood samples were collected from diabetic patients and normal controls after overnight fasting. All samples were immediately centrifuged and four aliquots of each serum were stored at -80°C until the time of assay.

sFas and sFasL serum concentrations

sFas was determined using sFas enzyme-linked immunosorbent assay (ELISA) kit (Research & Diagnostics Systems, Minneapolis, U.S.A.), with a sensitivity of 20 pg/ml in serum. The value of sFas in normal human serum is of 9.4 ng/ml. sFas was tested in all

| Table I. Characteristics of normal controls and diabetic patients. |
|---------------------------------|-----------------|-----------------|-----------------|

<table>
<thead>
<tr>
<th></th>
<th>normal controls (NC)</th>
<th>diabetic patients</th>
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<tr>
<td></td>
<td>without complication</td>
<td>with retinopathy</td>
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<td></td>
<td>(DW)</td>
<td>(DR)</td>
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<td>n</td>
<td>15</td>
<td>18</td>
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<tr>
<td>Men/women</td>
<td>8/7</td>
<td>10/8</td>
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<td>Age range (years)</td>
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<tr>
<td>&lt; 40 n =</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td>40-60 n =</td>
<td>8</td>
<td>11</td>
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<tr>
<td>&gt; 60 n =</td>
<td>—</td>
<td>1</td>
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<tr>
<td>mean ±SD</td>
<td>38.2 ± 10.5</td>
<td>44.7 ± 13.4 (¹)</td>
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<td>Hba1c (%)</td>
<td>5.2 ± 0.9</td>
<td>10.6 ± 2.0 (#)</td>
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<td>Plasma glucose concentration (mmol/L)</td>
<td>4.9 ± 0.4</td>
<td>13.4 ± 4.2 (#)</td>
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<tr>
<td>Type 1/Type 2 diabetes</td>
<td>—</td>
<td>6/12</td>
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<tr>
<td>Duration of diabetes (years):</td>
<td></td>
<td></td>
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<tr>
<td>&lt; 5 n =</td>
<td>—</td>
<td>9</td>
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<td>5-10 n =</td>
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<td>8</td>
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<tr>
<td>10-20 n =</td>
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¹ p < 0.05: a significant difference was present when the age of normal controls and diabetic patients with a predominant neuropathy was compared.

¹ no significant difference for these characteristics as compared among the diabetic patients.
sFasL was assayed using sFasL ELISA kit (Medical & Biological Laboratories, Nagoya, Japan.) with a sensitivity of 0.1 ng/ml in serum. The value of sFasL in normal human serum is less than 0.1 ng/ml, according to the manufacturer.

sFasL was tested in fifteen normal controls and in thirty diabetic patients (DW = 12, DR = 9, DN = 9; type1 = 9, type 2 = 21; men = 16, women =14). All procedures were performed at room temperature, according to the manufacturer’s instructions. Each sample and standard protein were assayed in duplicate. Optical density at 450 nm for sFasL and sFas was measured with a spectrophotometric microtitr plate reader (Labsystems iEMS Reader, Helsinki, Finland). A standard curve obtained with sFasL or sFas samples provided with the kit was used to determine the sFasL and sFas concentration in each sample.

Statistical analysis

sFas and sFasL levels in serum from normal controls were compared with those of diabetic patients by one-way analysis of variance and Bonferroni - Student test. A two-way analysis of variance was also carried out according to sex and group. Results are expressed as mean ± SD. A value of $p < 0.05$ was accepted as statistically significant.
RESULTS

sFasL serum levels

The values obtained in serum from normal controls were less than 0.1 ng/ml and considered as normal. The same results were observed in sera from all groups of diabetic patients and also considered as normal.

sFas serum levels (Fig. 1 and 2)

sFas serum levels were 7.1 ± 1.1 ng/ml in normal controls, a value similar to the normal value given by the manufacturer. sFas serum levels in diabetic patients with a predominant retinopathy were not significantly different when compared with normal controls (8.7 ± 1.9 ng/ml vs 7.1 ± 1.1 ng/ml, p > 0.05). In contrast, sFas serum levels in diabetic patients with a predominant neuropathy were significantly increased when compared with normal controls (13.5 ± 3.6 ng/ml vs 7.1 ± 1.1 ng/ml, p < 0.001), and with diabetic patients without complications (vs 9.1 ± 1.8 ng/ml, p < 0.001) or with retinopathy (vs 8.7 ± 1.9 ng/ml, p < 0.001). Also, sFas serum levels in diabetic patients without complications were significantly increased when compared with normal controls (9.1 ± 1.8 ng/ml vs 7.1 ± 1.1 ng/ml, p < 0.02).

Since Seishima et al. [14] reported that the mean value of sFas was significantly higher in men than in women, we compared sFas serum levels in men and women among each group of diabetic patients and normal controls. We observed that sFas levels were higher in sera from men than those from women only in the group of diabetic patients with a predominant neuropathy (14.4 ± 3.2 ng/ml vs 11.3 ± 4.1 ng/ml, p < 0.05). We also compared sFas serum levels only among men of all groups and we observed that sFas serum levels in diabetic patients with a predominant neuropathy were significantly increased when compared with normal controls (14.4 ± 3.2 ng/ml vs 8.4 ± 2.2 ng/ml, p < 0.001), and with diabetic patients without complications (vs 9.3 ± 2.0 ng/ml, p < 0.001) or with retinopathy (vs 8.7 ± 1.9 ng/ml, p < 0.001).

DISCUSSION

We measured sFasL and sFas levels in serum from diabetic patients with or without complications and from normal controls. sFasL serum levels were found to be normal in all diabetic patients and in normal controls. Some comments may be done to explain this situation:

1) FasL might be cross-linked with Fas or sFas, and consequently, its free soluble form not available in the serum.

2) Antibodies used in this work might not recognize the sFasL epitope present in the sera from diabetic patients and normal controls. Recent criticisms about the detection of cell surface FasL suggested that antibodies used to recognize FasL were not always appropriate for the detection of this protein [15].

3) FasL might not be present at the surface of producing cells and Fas mediated apoptosis observed in pancreatic β cells might be independent from the Fas-FasL interaction, as observed in hepatocytes where it has been reported that bile salts induce apoptosis via direct activation of Fas [16].

![Fig. 1. sFas serum levels in diabetic patients with a predominant neuropathy (DN) as compared with normal controls (NC), diabetic patients without complications (DW), diabetic patients with a predominant retinopathy (DR): *** p < 0.001, DN vs NC, DW or DR. sFas serum levels in diabetic patients without complications as compared with normal controls: * p < 0.02 DW vs NC. sFas serum levels in diabetic patients with retinopathy as compared with normal controls: # p = 0.08, DR vs NC (NS). Values are means ± SD.](image1)

![Fig. 2. Individual values of sFas serum levels (N < 9.4 ng/ml) in each groups of diabetic patients and normal controls.](image2)
sFas serum levels were found to be normal in diabetic patients with a predominant retinopathy. Fas and FasL are expressed on the surface of pericytes and endothelial cells (EC) which were however reported to be resistant to Fas-mediated apoptosis. But in human cultured EC, high glucose concentrations can trigger apoptosis, and the Fas pathway could be activated by oxidized low density lipoproteins [17]. Some explanations can be given:

1) Similarly to suggested above, antibodies could not recognize the sFas isoform released by pericytes or EC.

2) Through the effects of factor(s) able to sensitize Fas by its ligand, an autocrine/paracrine Fas-FasL interaction could be a possible mechanism for an autoimmune destruction of EC and pericytes, without any sFas or sFasL serum level increase. Such a possibility was suggested for other cells such as pancreatic β cells, thyrocytes and T lymphocyte cells, where an autocrine cell suicide was mediated by Fas/FasL interaction [18].

In contrast, sFas serum levels were significantly increased in diabetic patients with a predominant neuropathy. Clinical examination and biochemical characteristics of our diabetic patients did not show any major complication except for the neuropathy to account for the sFas increase (Table 1). Indeed, in all groups of diabetic patients, levels of HbA1c was > 10% and considered as more elevated since usually normal value accepted is < 6.5%. Plasma glucose concentration also was elevated, without a significant difference among the diabetic groups. Regarding the duration of diabetes, the majority of patients had a diabetes for five to ten years. Some patients had a diabetes for less than five years, even in the group with neuropathy. This is not surprising since, as opposed to a widespread belief, polyneuropathy is not a late complication of diabetes, but may occur early in the disease. However in this group, the number of patients with a duration of diabetes higher than ten years was more important than in other groups; nevertheless, the value of sFas obtained in each of these patients was within the range of values obtained in the other diabetic patients with neuropathy. The average age of all diabetic patients were quite homogeneous, except for a significant difference between the age of normal controls and that of the diabetic patients with neuropathy. Finally, in agreement with Seishima et al. [14], we showed that sFas serum levels were significantly higher in men than in women, but only among the group of diabetic patients with neuropathy.

Two possible mechanisms could explain the increase of sFas: a stimulation of the Fas gene yielding to sFas by alternative Fas mRNA splicing, or an overexpression of the Fas receptor and consequently an increase in sFas. But the place where Fas is overexpressed in cells is still unclear. Biopsies from diabetic patients with neuropathy showed a loss of neuronal fibers, demyelinative lesions, alterations of microvascularisation, and inflammatory infiltrates of mononuclear cells [19]. Two types of cells may be involved: peripheral nerve cells and peripheral blood mononuclear (PMN) cells.

— Peripheral nerve cells: Fas-mediated apoptosis observed in vitro in oligodendrocytes and Schwann cells has been suggested to be involved in the demyelination of these cells [20, 21]. It was reported that proinflammatory Th1 cytokine could upregulate FasL or Fas on the surface of activated Schwann cells, also considered as immunocompetent cells [22]. In addition, a recent publication reported that diabetic peripheral neuropathy is associated with activation of apoptosis [23]. We hypothesize that an overexpression of one molecule of the Fas system in peripheral nerve cells could promote cross-linking with Fas molecule expressed on infiltrating cytotoxic mononuclear cells and trigger apoptotic death of the nerve cells. Cytokines and the factors usually involved in the pathogenesis of diabetic neuropathy could be also involved in this dysregulation of the Fas system. A defect in neutrotrophic factors essential for the protection of neurons against apoptosis, such as the Nerve Growth Factor (NGF) or Insulin-like Growth Factor (IGF), also may be possible [24, 25].

— Peripheral blood mononuclear cells: When they are activated, PMN cells can overexpress Fas and FasL and secrete cytokines involved in cell injury or death. In recently diagnosed type 1 diabetic patients, activated circulating cytotoxic Tc1 cells were observed in the peripheral blood [26]. In both types of diabetes, polymorphonuclear neutrophils were involved in the development of late complications of diabetes [27]. We suggest that:

1) The sFas serum level increase may be also the result of a continuous activity of PMN cells remaining after insulitis or other inflammatory process.

2) It is possible that a genetic defect only present in diabetic patients with neuropathy leads to the predominance of alternatively spliced Fas gene transcripts after PMN cells activation by different factors usually involved in diabetic complications, as suggested in systemic lupus erythematosus [28].

sFas serum levels were also significantly increased in diabetic patients without complications compared to normal controls. We propose that the sFas serum level increase might appear before clinical symptoms in diabetic patients who will present diabetic neuropathy later on but this result needs to be confirmed on a larger series of patients and by a follow-up of the patients of this study.

In conclusion, we showed in this prospective study that sFas serum levels were increased in diabetic patients with a predominant neuropathy and we suggest that a dysregulation of the Fas system may be involved. This dysregulation is still to be analyzed by complementary studies in order to treat or prevent the
occurrence of this complication which is present with a similar frequency in both type 1 and type 2 diabetes.

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