Accelerated Production of Nucleosome in Cultured Human Mononuclear Cells in Untreated Graves' Disease

Hideo Hara, Ryuji Sato and Yoshio Ban

The 3rd Department of Internal Medicine, Showa University School of Medicine, Tokyo 142-8666, Japan

Abstract. The apoptosis of lymphocytes, which occurs in autoimmune diseases, is usually induced by the Fas/Fas ligand system. As the assay of nucleosomes produced by apoptotic cells can be used to quantitate apoptosis, we evaluated nucleosome and soluble Fas ligand (sFasL) levels of cultured mononuclear cells to clarify the apoptosis of mononuclear cells in patients with autoimmune thyroid diseases by enzyme-linked immunosorbent assay. Nucleosome levels of cultured mononuclear cells in patients with untreated Graves’ disease were significantly higher (3.27±2.90 U/ml) than those of control subjects (1.39±0.24 U/ml) and euthyroid patients with treated Graves’ disease (1.53±0.33 U/ml). Nucleosome levels of cultured mononuclear cells were positively correlated with sFasL levels (r=0.544, p<0.01). It is therefore likely that increased sFasL levels elicit apoptosis of these cells in untreated Graves’ disease.

Key words: Nucleosome, Graves’ disease, Cultured mononuclear cell


The form of cell death called apoptosis governs the regulation of cell number and cell differentiation by inducing a series of morphological changes such as nuclear condensation and cell surface blebbing leading to the formation of apoptotic bodies [1]. Apoptosis is induced by Fas antigen in conjunction with Fas ligand, and soluble Fas is also known to interact with the system [2–4]. The Fas/Fas ligand system plays an important role in apoptosis [5, 6], and soluble Fas (sFas) and Fas ligand (sFasL), two proteins generated by alternative splicing [2, 7, 8] which lack the transmembrane domain, are thought to be related to apoptosis. The former, sFas, suppresses apoptosis while the latter, sFasL, accelerates it. When a cell enters the apoptotic pathway there is an endonuclease-mediated digestion of the exposed DNA linker regions between the histones in chromatin, while cytochrome c can be released from mitochondria into the cytosol in response to apoptotic signals [9–12], which has been shown to induce cell apoptosis through the activation of caspase [10, 13, 14]. Since endonuclease-mediated nucleosome excision is observable as a DNA ladder in agarose, nucleosome assay can be used to quantitate apoptosis [15, 16].

Apoptosis of lymphocytes, a process which plays a critical role in the positive and negative selection of immature T cells and pre-B lymphocytes in the thymus, has been observed in autoimmune diseases such as systemic lupus erythematosus [16, 17]. Furthermore, the functional properties of T clones from peripheral blood lymphocytes in autoimmune thyroid diseases and the increase of apoptotic neutrophils in systemic lupus erythematosus have been demonstrated [18, 19].

Therefore, to clarify the prevalence of apoptosis of mononuclear cells in autoimmune thyroid diseases, we studied the levels of nucleosome, sFas and sFasL in culture supernatants of mononuclear cells in patients with Graves’ disease and Hashimoto’s thyroiditis.
Subjects and methods

Peripheral blood samples were obtained from 12 healthy control subjects (2 males, 10 females, mean age 45.9 years), 17 patients with untreated Graves' disease (2 males, 15 females, mean age 45.6 years), 11 euthyroid patients with Graves' disease treated by methimazole for over 6 months (3 males, 8 females, mean age 45.7 years) and 7 patients with untreated Hashimoto's thyroiditis (7 females, mean age 53.8 years), from whom informed consent was obtained. The samples were drawn into a lymphocyte separation media (CPT™ Cell Preparation Tube, Sodium Heparin Gel and Density Gradient Media, Becton Dickinson, Franklin Lakes, U.S.A.). At the same time, serum samples were obtained to be measured for serum free thyroxine (FT₄), free triiodothyronine (FT₃), thyroid stimulating hormone (TSH) and TSH receptor antibody (TRAb).

Mononuclear cells were isolated from the media by centrifugation for 30 min at 400 × g. The cells were washed twice with 0.9% saline solution and suspended in GIT medium (Growth factor in serum + Insulin, Transferrin, Ethanolamine, Selenite + Daigo's medium, Wako Co., Osaka, Japan) at a concentration of 1 × 10⁶ cells/ml. One ml aliquots of the medium were added to tissue culture clusters (Costar Co., Cambridge, MA, U.S.A.) and cultured in 5% CO₂ and 95% air at 37°C for 24 hours. Nucleosome, sFas and sFasL levels were measured in aliquots of the supernatants after centrifugation. Only in untreated Graves' disease were cytochrome c and caspase-3 evaluated in the supernatants.

A portion of the cultured mononuclear cells was used for DNA analysis. DNA extracted from the cells, in total comprising multiples of 123 base pairs, was electrophoresed (DNA ladder detection kit, Wako Co., Osaka, Japan) on 1.5% agarose gel. The gel was stained briefly with SYBR™ Green I Working Solution (Molecular Probes Inc., OR, U.S.A.) and photographed under ultraviolet transillumination. The survival rates of cultured mononuclear cells from 4 control subjects, 10 patients with untreated Graves' disease and 3 patients with Hashimoto's thyroiditis were measured by staining the cells with trypan blue stain (Gibco Laboratories, NY, U.S.A.) after culture.

Enzyme-linked immunosorbent assays were used for nucleosome (Nucleosome ELISA, Oncogene Research Products, Cambridge, MA, U.S.A.), sFas (sFas ELISA Kit, MBL Co., Osaka, Japan), sFasL (sFas Ligand ELISA Kit, MBL Co., Osaka, Japan), caspase-3 (Human Active Caspase-3 Immunoassay, R&D systems, Minneapolis, U.S.A.) and cytochrome c (Human Cytochrome c immunoassay, R&D systems, Minneapolis, U.S.A.). Minimal detectable levels of nucleosome, sFas and sFasL were 0.05 U/ml, 0.01 ng/ml and 0.002 ng/ml, respectively. Interassay and intraassay coefficients of variation were 5.3% and 4.9% for nucleosome, 4.2% and 5.2% for sFas, and 5.3% and 8.2% for sFasL, respectively. The methods used chemiluminescent immunoassay for TSH (Amerlite TSH 30 kit), RIA for FT₄ (Amerlex M FT₄ kit) and FT₃ (Amerlex M FT₃ kit), and radioreceptor assay for TRAb (Cosmic Co).

The results are expressed as mean ± standard deviation (SD). Statistical significances of differences between the patients and control subjects were calculated by ANOVA, and the correlations between nucleosome and sFas, sFasL, caspase-3 and cytochrome c levels in cultured mononuclear cells were determined by Fisher's test. P-value of < 0.05 was considered to be statistically significant.

Results

1. DNA ladder analysis

Agarose gel electrophoresis of extracted DNA from cultured mononuclear cells from a control subject, 2 patients with Graves' disease and 2 patients with Hashimoto's thyroiditis showed a ladder pattern consisting of multiples of about 123 base pairs (Fig. 1). There was no difference of the pattern between Graves' disease and Hashimoto's thyroiditis.

2. Correlation between survival rate and nucleosome levels in the supernatants of cultured mononuclear cells.

In 13 patients and 4 normal control subjects, the survival rates were measured by staining cultured mononuclear cells with trypan blue stain. There was a significant negative correlation between the survival rates and nucleosome levels in the supernatants of cultured mononuclear cells, as shown in Fig. 2 (r = -0.907, P < 0.001).
3. Nucleosome, sFas, sFasL, caspase-3 and cytochrome c levels in culture supernatants of mononuclear cells in patients with Graves' disease, Hashimoto's thyroiditis and healthy control subjects.

Nucleosome, sFas and sFasL levels in the supernatants in patients with Graves' disease, Hashimoto’s thyroiditis and control subjects are shown in Table 1. Nucleosome levels were significantly higher in the supernatants in patients with untreated Graves' disease than those in control subjects and in patients with euthyroid Graves' disease (P<0.01 and P<0.02, respectively). sFasL levels were remarkably higher in patients with untreated Graves' disease than those of control subjects and patients with Hashimoto’s thyroiditis (P<0.01 and P<0.02, respectively). There were no significant differences of nucleosome and sFasL in the supernatants between control subjects and patients with Hashimoto’s thyroiditis.

Table 1. Nucleosome, sFas and sFasL levels of culture supernatants of mononuclear cells in patients with Graves’ disease, Hashimoto’s thyroiditis, and control subjects.

<table>
<thead>
<tr>
<th>Patients</th>
<th>No.</th>
<th>Age (y)</th>
<th>TSH (mIU/l)</th>
<th>FT4 (ng/dl)</th>
<th>FT3 (pg/ml)</th>
<th>TRAb (%)</th>
<th>Nucleosome (U/ml)</th>
<th>sFas (ng/ml)</th>
<th>sFasL (ng/ml)</th>
</tr>
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<tbody>
<tr>
<td>Control untreated</td>
<td>12</td>
<td>46.3</td>
<td>±4.4</td>
<td>1.20</td>
<td>1.22</td>
<td>±0.18</td>
<td>3.73 ±0.38</td>
<td>3.3 ±1.1</td>
<td>1.39 ±0.24</td>
</tr>
<tr>
<td>Graves’ disease</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>untreated</td>
<td>17</td>
<td>44.0</td>
<td>±18.5</td>
<td>5.93*</td>
<td>±2.03</td>
<td>13.89*</td>
<td>30.5* ±2.90</td>
<td>3.27* ±2.90</td>
<td>0.018 ±0.010</td>
</tr>
<tr>
<td>treated</td>
<td>11</td>
<td>41.0</td>
<td>±11.8</td>
<td>1.30</td>
<td>±0.24</td>
<td>3.53</td>
<td>5.5 ±3.7</td>
<td>1.53* ±0.33</td>
<td>0.009 ±0.003</td>
</tr>
<tr>
<td>Hashimoto’s</td>
<td>7</td>
<td>47.8</td>
<td>±17.6</td>
<td>11.97</td>
<td>±0.15</td>
<td>2.83</td>
<td>1.36 ±0.65</td>
<td>0.015 ±0.009</td>
<td>0.023* ±0.015</td>
</tr>
</tbody>
</table>

Data are expressed as means ±SD. Significant difference by ANOVA.
* P<0.01 vs. control; ** P<0.02 vs. control; † P<0.02 vs. untreated Graves’ disease; ‡ P<0.05 vs. untreated Graves’ disease.
There were no differences in sFas levels between untreated Graves' disease and control subjects, or between Hashimoto’s thyroiditis and control subjects. Cytochrome c levels in the supernatants in patients with untreated Graves' disease (0.712±0.410 ng/ml), but not caspase-3 levels, were significantly higher than cytochrome c levels of control subjects (0.304±0.064 ng/ml) (P<0.01).

4. Correlation between nucleosome, cytochrome c, caspase-3, sFas and sFasL levels in culture supernatants of mononuclear cells.

There was a significant positive correlation between supernatant levels of sFasL and nucleosome in patients with Graves' disease, patients with Hashimoto’s thyroiditis and control subjects (r = 0.544, P<0.01), but there was no correlation between nucleosomes and sFas, or the serum thyroid hormones in them.

Nucleosome levels were significantly correlated with sFasL levels in patients with Graves' disease (Fig. 3; r = 0.476, P<0.05), and in patients with untreated Graves' disease (r = 0.547, P<0.05). Positive correlations were observed between caspase-3 and sFasL (r = 0.697, P<0.02), and between caspase-3 and cytochrome c (r = 0.645, P<0.05) in culture supernatants in untreated Graves' disease.

**Fig. 3.** Correlation between nucleosome and sFasL levels in cultured mononuclear cells from patients with untreated Graves' disease.

**Discussion**

Nucleosomes are derived from apoptotic cells. The nucleosome levels were negatively correlated with survival rate of cultured mononuclear cells, and agarose gel electrophoresis of extracted DNA from cultured mononuclear cells showed a ladder pattern that demonstrated their apoptotic activity [20]. Therefore, we measured sFas, sFasL and nucleosome levels in culture supernatants of mononuclear cells in patients with Graves’ disease and Hashimoto’s thyroiditis to clarify how the apoptosis of mononuclear cells is related to these two diseases.

The apoptosis reported in thyroid disease was observed in the thyroid tissue [21], but there has been no report on apoptosis of mononuclear cells in autoimmune thyroid diseases detected by measurement of nucleosome levels of cultured mononuclear cells. Here we studied the production of nucleosomes from cultured mononuclear cells in autoimmune thyroid diseases.

In this study we observed an increase of nucleosome and sFasL levels, and demonstrated a significant positive correlation between nucleosome and sFasL in culture supernatants of mononuclear cells in patients with untreated Graves’ disease. Although there was no correlation between serum thyroid hormones and these levels. These results suggested that the increased sFasL levels in patients with untreated Graves’ disease, but not thyroid hormones, play a critical role in cell death of mononuclear cells, which shows that the increase levels of sFasL are related to the acceleration of the apoptotic pathway. According to the nucleosome and sFasL levels in the supernatant, mononuclear cells in patients with untreated Graves’ disease may be prone to apoptotic cell death. This finding was supported by the report that thyroid-infiltrating lymphocytes expressed decreased levels of the antiapoptotic molecule Bcl-2 in Graves’ disease compared to the high levels in Hashimoto’s thyroiditis, and the opposite pattern in thyrocytes [22], but that the sFas levels interacting in the apoptotic pathway were not so high [23]. The positive correlations between sFasL and caspase-3, and between cytochrome c and caspase-3 in untreated Graves’ disease suggested that the apoptotic pathway in Graves’ disease may be accelerated through the Fas/FasL system, as the system may activate caspase which induces cellular apoptosis through the release
of cytochrome c into cytosol [9, 10, 12]. It is supposed that the increased levels of nucleosomes and sFasL levels in the supernatants in patients with untreated Graves’ disease might be affected by TSH or TRAb [23]. Furthermore, it is speculated that an imbalanced apoptosis of mononuclear cells which produce certain kinds of cytokines [24] or immunoglobulins that are related to the secretion of thyroid hormones from thyrocytes may exist in patients with untreated Graves’ disease. These possibilities need to be further investigated in the supernatants using cultured mononuclear cells with higher levels of Bcl-2, Bax or sFasL, and measuring cytokines or immunoglobulins [25]. It is thought that mononuclear cells in patients with untreated Graves’ disease might express FasL in vitro and may have a much greater involvement in peripheral NK cell activity than in mononuclear cell activity in the thyroid gland [26, 27]. It is further speculated that, in untreated Graves’ disease, cell differentiation and cell number might be regulated by an acceleration of endogenous endonuclease activity which increases nucleosomes to much higher levels [28].

Nucleosome, sFas and sFasL levels in patients with Hashimoto’s thyroiditis were not significantly different from those of control subjects. To explain the lack of difference in nucleosome levels between patients with Hashimoto’s thyroiditis and control subjects, it was speculated that mononuclear cells from peripheral blood in Hashimoto’s thyroiditis may undergo apoptosis at about the same rate as those from control subjects. They may produce much less sFasL than untreated Graves’ disease and Bcl-2, which thyroid-infiltrating lymphocytes expressed high levels of, thus inhibiting the apoptosis of mononuclear cells in Hashimoto’s thyroiditis; that is the Fas/FasL system may possibly induce apoptosis of thyrocytes in Hashimoto’s thyroiditis [22], even though its nucleosome levels might not be so high. We hypothesized that in Graves’ disease apoptosis of mononuclear cells might occur more in peripheral blood than in thyrocytes and that in Hashimoto’s thyroiditis it might be opposite.

We concluded that patients with untreated Graves’ disease might have high levels of nucleosome and sFasL in cultured mononuclear cells which produced much more sFasL and nucleosomes than those of control subjects. Our results suggested that the acceleration of peripheral mononuclear cells in patients with untreated Graves’ disease are an important factor related to apoptosis pathway and the Fas/Fas ligand system.

References