Production of 8-OHdG and Cytochrome c by Cultured Human Mononuclear Cells in Patients with Autoimmune Thyroid Disease

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Abstract. Since oxidative stress is related to autoimmune thyroid disease, we studied the production of 8-hydroxy-2'-deoxyguanosine (8-OHdG) and cytochrome c by culture of mononuclear cells from patients with Graves' disease and Hashimoto's thyroiditis. In patients with untreated Graves' disease, 8-OHdG and cytochrome c levels in culture supernatant of mononuclear cells were significantly higher than those of healthy control subjects, while the cytochrome c levels were significantly higher in patients with untreated Graves' disease and Hashimoto's thyroiditis than those of control subjects. Significant correlations between 8-OHdG and FT4i, and cytochrome c were found. These results indicated that thyroid function has a potent influence on oxidative stress.

Key words: 8-OHdG, Cytochrome c, Autoimmune thyroid disease

SUPEROXIDES, which damage cellular DNA, are constantly generated in cellular mitochondria to produce energy and to attack external substances in human cells. The damage by oxygen radicals may contribute mainly to aging, cancer, and heart disease amongst others [1, 2]. Numerous defense systems protect cellular macromolecules against superox- ides, but the rate of damage to DNA, protein, and lipids is high. The imbalance of the antioxidant defense system and oxidative damage by superox- ides has been shown in autoimmune diseases [3, 4].

When deoxyguanosine composing DNA is oxidized, it is converted into 8-hydroxy-2'-deoxyguanosine (8-OHdG). Thus 8-OHdG is one of the most useful markers for the evaluation of oxidative DNA damage and oxidative stress [5].

Human somatic cytochrome c is a 15 kDa poly- peptide that takes part in both oxidative phosphorylation and apoptosis [6]. In response to apoptotic signals, cytochrome c can be released from mitochondria into the cytosol [7-9].

The 8-OHdG and cytochrome c levels of cultured mononuclear cells in the patients were measured to study the relationship between oxidative stress and autoimmune thyroid disease, and the degree of oxidative stress in patients with Graves' disease and Hashimoto’s thyroiditis.

Subjects and methods

Blood samples were drawn into lymphocyte separation media (CPT™ cell preparation tube, sodium heparin gel and density gradient media) from 12 healthy control subjects (1 male, 11 female, mean age 46.3 years), from 17 patients with untreated Graves' disease (3 male, 14 female, mean age 44.0 years), from 12 patients with treated euthyroid Graves' dis-
ease (3 male, 9 female, mean age 41.0 years) and from 7 patients with untreated Hashimoto’s thyroiditis (female, mean age 47.8 years), from whom informed consent for participation was obtained. There was no significant difference in the mean age between patients with Graves’ disease, Hashimoto’s thyroiditis and control subjects. At the same time the 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 heparinized venous blood by centrifugation for 30 min at 400 × g. Cells were washed twice with 0.9% saline solution, suspended in GIT medium (growth factor in serum + insulin, transferrin, ethanalamine, selenite + Daigo’s medium) at a concentration of 1 × 10^6 cells/ml, each 1 ml of which was poured into tissue culture clusters (Data Packaging Co.), and cultured in 5% CO2 and 95% air at 37°C for a day. Samples to be measured were taken from the well a day after commencement of culture. Aliquots of the supernatant after centrifugation were measured for 8-OHdG, cytochrome c, and nuclear matrix protein (NMP). Survival rates of cultured mononuclear cells from 4 control subjects and 13 patients were measured by staining the cells with trypan blue stain (Gibco Laboratories, New York, NY) after culture.

8-OHdG was determined with an enzyme-linked immunosorbent assay (ELISA) kit (Japan Institute for the Control of Aging, Shizuoka, Japan). Cytochrome c was measured with an enzyme immunoassay (Human Cytochrome c immunoassay, R&D systems, Minneapolis). The methods used were ELISA for NMP (Nuclear Matrix Protein ELISA, Oncogene Research Products, Cambridge), chemiluminescent immunoassay for TSH (Amerlite TSH 30 kit), RIA for FT3 (Amerlex M FT3 kit) and FT4 (Amerlex M FT4 kit), and radioreceptor assay for TRAb (Cosmic Co.). Minimal detectable levels of 8-OHdG and cytochrome c were 0.1 ng/ml, and 0.15 ng/ml, respectively. Interassay and intraassay coefficients of variation were 4.7% and 4.3% for 8-OHdG, and 3.9% and 4.1% for cytochrome c, respectively.

Data are expressed as mean±SD. Fisher’s test was used to compare differences and to determine the correlations between levels of 8-OHdG, NMP and cytochrome c in the supernatants of cultured mononuclear cells, serum thyroid hormones and TRAb.

### Results

1. Survival rates of cultured mononuclear cells.

   After the commencement of culture, the survival rates of mononuclear cells were over 95%, and there was no difference in the rate between control subjects and the patients.

2. 8-OHdG and cytochrome c levels of culture supernatants of mononuclear cells in patients with Graves’ disease, Hashimoto’s thyroiditis, and control subjects.

   8-OHdG and cytochrome c levels of culture supernatants of mononuclear cells in patients with Grave’s disease, Hashimoto’s thyroiditis, and control subjects.

<table>
<thead>
<tr>
<th>Patients</th>
<th>No.</th>
<th>Age</th>
<th>TSH (mU/l)</th>
<th>FT4 (ng/dl)</th>
<th>FT3 (pg/ml)</th>
<th>TRAb (%)</th>
<th>8-OHdG (ng/ml)</th>
<th>Cytochrome c (ng/ml)</th>
<th>NMP (IU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control subjects</td>
<td>11</td>
<td>46.3</td>
<td>1.20</td>
<td>1.22</td>
<td>3.73</td>
<td>3.3</td>
<td>3.00</td>
<td>0.30</td>
<td>16.6</td>
</tr>
<tr>
<td>± 4.4</td>
<td>±0.18</td>
<td>±0.18</td>
<td>±0.38</td>
<td>±1.1</td>
<td>±0.89</td>
<td>±0.06</td>
<td>±2.7</td>
<td>26.6*</td>
<td></td>
</tr>
<tr>
<td>Grave’s 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>14</td>
<td>44.0</td>
<td>&lt;0.02*</td>
<td>5.90*</td>
<td>14.35*</td>
<td>30.5*</td>
<td>6.98*</td>
<td>0.72*</td>
<td>26.6*</td>
</tr>
<tr>
<td>±18.5</td>
<td>±2.03</td>
<td>±2.03</td>
<td>±6.92</td>
<td>±25.8</td>
<td>±3.89</td>
<td>±0.41</td>
<td>11.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Treated</td>
<td>12</td>
<td>41.0</td>
<td>2.10</td>
<td>1.30</td>
<td>3.53</td>
<td>5.5</td>
<td>3.72</td>
<td>0.37</td>
<td>19.1</td>
</tr>
<tr>
<td>±11.8</td>
<td>±1.37</td>
<td>±1.37</td>
<td>±0.24</td>
<td>±3.7</td>
<td>±1.31</td>
<td>±0.11</td>
<td>±4.46</td>
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<td></td>
</tr>
<tr>
<td>Hashimoto’s thyroiditis</td>
<td>7</td>
<td>47.8</td>
<td>11.97</td>
<td>1.02</td>
<td>2.83</td>
<td>2.59</td>
<td>0.42**</td>
<td>0.12</td>
<td>18.9</td>
</tr>
<tr>
<td>±17.6</td>
<td>±9.19</td>
<td>±9.19</td>
<td>±0.53</td>
<td>±1.71</td>
<td>±1.71</td>
<td>±6.9</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Data are expressed as mean±SD. Significant difference from control subjects was determined by Fisher’s test. p<0.01; *, p<0.02; **
The supernatants of mononuclear cells in patients with Graves' disease, Hashimoto's thyroiditis, and control subjects are shown in Table 1. 8-OHdG and cytochrome c levels of the supernatants in patients with untreated Graves' disease were significantly higher than in control subjects (p<0.01), in patients with euthyroid Graves' disease (p<0.02) and in patients with Hashimoto's thyroiditis (p<0.05).

In Hashimoto's thyroiditis, cytochrome c levels of the supernatants were significantly higher than those in control subjects (p<0.02).

3. Correlations between 8-OHdG, cytochrome c, NMP of culture supernatants of mononuclear cells, serum thyroid hormones and TRAb.

Cytochrome c levels in culture supernatants of mononuclear cells were positively correlated with 8-OHdG (r=0.394, p<0.02) (Fig. 1), NMP (r=0.650, p<0.01) (Fig. 2), and serum FT4 levels (r=0.475, p<0.01) (Fig. 3) except for Hashimoto's disease. A positive correlation between 8-OHdG levels in the supernatants and serum FT4 levels was observed (r=0.408, p<0.01) except for Hashimoto's disease. There was no evident correlation between 8-OHdG and TSH, or 8-OHdG and FT3 or 8-OHdG and TRAb.

In Hashimoto's thyroiditis, cytochrome c levels were not remarkably correlated with 8-OHdG, NMP and serum FT4.

Discussion

Oxidative stress usually activates caspase which induces cellular apoptosis through the release of cytochrome c into cytosol [7, 8]. Abnormalities in the human immune system induced by apoptosis of T and B cells are related to the cause of autoimmune
diseases [10-12]. We reported that serum superoxide dismutase, an antioxidant that acts as a scavenger, is increased in patients with Graves’ disease [13]. The purpose of this study was to determine the levels of 8-OHdG, which is a useful marker for oxidative damage to cells, and, those of cytochrome c, which is released into cytosol in apoptosis, in culture supernatant of mononuclear cells in autoimmune thyroid diseases.

8-OHdG and cytochrome c levels in culture supernatant of mononuclear cells in patients with untreated Graves’ disease were significantly higher than those of patients with euthyroid Graves’ disease, Hashimoto’s thyroiditis and control subjects. Cytochrome c in culture supernatants of mononuclear cells was positively correlated with 8-OHdG, NMP to quantitate cell death [14] and serum FT4. These results suggested that mononuclear cells of patients with untreated Graves’ disease were affected by oxidative stress in vitro, and that thyroid hormone might mediate the production of 8-OHdG, which supports the view of Mihara et al. who reported that thyroid hormones produce reactive oxygen species [15]. Furthermore, the increased levels of thyroid hormones might contribute to the high levels of cytochrome c and 8-OHdG in the supernatant in Graves’ disease. It was thought that both 8-OHdG and thyroid hormone could accelerate to release cytochrome c into cytosol in mononuclear cells in Graves’ disease. From the point of view of cell apoptosis, cytochrome c released by superoxides into cytosol has been shown to induce cell apoptosis through the activation of caspase [8, 16, 17]. That report and our results suggested that certain kinds of mononuclear cells of patients with Graves’ disease which had been exposed to high serum levels of thyroid hormones could be disposed to apoptotic cell death in vitro that was affected by oxidative stress, and might be closely related to 8-OHdG as well as the Fas/FasL system. The report that antiapoptotic molecule Bcl-2 inhibited apoptosis of mononuclear cells which occurred as thyroid-infiltrating lymphocytes expressed decreased levels of Bcl-2 in Graves’ disease compared to the high levels in Hashimoto’s thyroiditis, while the opposite pattern was seen in thyrocytes which were shown to induce lymphocytes to apoptosis in Graves’ disease [18]. It is necessary to study further which of the lymphocytes are being induced to apoptosis in Graves’ disease, and whether or not there is an increase in vitro in cytochrome c levels by culture of mononuclear cells of Graves’ disease with additive high levels of Bcl-2, antioxidants, and/or Bax.

Cytochrome c levels in culture supernatants of mononuclear cells in patients with Hashimoto’s thyroiditis, not 8-OHdG levels, were remarkably higher than those of control subjects, and were not positively correlated with NMP and serum FT4 levels. It was assumed that there was no significant difference in thyroid function between control subjects and patients with Hashimoto’s thyroiditis, and that 8-OHdG levels in culture supernatant of mononuclear cells in patients with Hashimoto’s thyroiditis were not different from those of control subjects. The reason why NMP levels in the supernatant in Hashimoto’s thyroiditis patients were not significantly different from those of control subjects is explained by the report that apoptosis of peripheral lymphocytes might be inhibited by the antiapoptotic molecules such as when the antiapoptotic molecule Bcl-2 levels were high [18], although in Hashimoto’s thyroiditis the Fas/FasL system may induce apoptosis of thyrocytes. It was supposed that the release of cytochrome c might be carried out by Fas/Fas ligand or TNF-α rather than 8-OHdG in culture supernatant in Hashimoto’s thyroiditis. These findings supported the results that cytochrome c levels in culture supernatant in Hashimoto’s thyroiditis were higher than those of control subjects. It is thus important to investigate the relationship of cytochrome c and Fas/Fas ligand in culture supernatant of mononuclear cells in Hashimoto’s thyroiditis.

Th1 and Th2 cells, amongst other lymphocytes that secrete cytokines, play an important role in the pathogenesis of autoimmune thyroid diseases [19]. It is necessary to further study which of the lymphocytes are induced to apoptosis in these diseases.

In summary, 8-OHdG levels in culture supernatant of mononuclear cells in patients with Graves’ disease were remarkably higher than those of control subjects and patients with Hashimoto’s thyroiditis, and the cytochrome c levels were significantly higher in patients with Graves’ disease and Hashimoto’s thyroiditis than those of control subjects. It can be thus concluded that oxidative stress and cytochrome c may have an important relation with Graves’ disease.
References


