Endothelial cells stimulate proliferation of human thyroid epithelial cells

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Abstract. The present study was undertaken to investigate cellular interactions between human thyroid epithelial cells (thyrocytes) and endothelial cells. Normal thyrocytes were cultured with either mitomycin C-treated endothelial cells or mitomycin C-treated human foreskin fibroblasts. The proliferative responses of thyrocytes were markedly stimulated by endothelial cells, but not by skin fibroblasts. The proliferative response of the thyrocytes obtained from patients with Graves' disease were similar to that of normal thyrocytes. Furthermore, the cell number of thyrocytes in endothelial cell-thyrocyte co-culture was markedly increased as compared with that in thyrocytes alone. The culture medium of endothelial cells only partly had any effect in the endothelial cell-thyrocyte co-culture experiment. Indomethacin, a cyclooxygenase inhibitor, did not increase the endothelial cells-induced thyrocyte proliferation. Furthermore, the increased proliferative response of thyrocytes stimulated by endothelial cells was not suppressed by heparin. These results suggest that endothelial cells increase thyrocyte proliferation, and that cell contact or extracellular matrix production by endothelial cells may play an important role in the proliferation of thyrocytes.

Thyroid enlargement is one of the most common manifestations of Graves' disease and Hashimoto's thyroiditis, however, the cause of the enlargement is uncertain. In addition to TSH and various antibodies, several humoral factors, including epidermal growth factors (EGF) (1-3), insulin-like growth factor-I (IGF-I) (4,5), fibroblast growth factor (FGF) (2) and interleukin 1 (IL-1) (6) have been reported to be growth factors on thyrocytes; the precise role of the factors as stimulators of thyroid growth, however, is not known. Thyroid tissue from patients with autoimmune thyroid diseases is characterized by prominent neovascularization and infiltration of mononuclear cells. The neovascularization is critically dependent on the local proliferation of endothelial cells (7). Proliferation of endothelial cells in experimental models of thyroid hyperplasia begins earlier than that of thyroid follicular cells (8). From the above findings, it appears likely that endothelial cells may play a critical role in the proliferation of thyrocytes.

The present study was undertaken to determine whether endothelial cells could stimulate proliferation of human thyrocytes.

Materials and Methods

Preparation of endothelial cells
Endothelial cells were obtained from human umbilical veins by an enzymatic digestion technique according to previously described methods (9,10). In brief, umbilical veins were cannulated and perfused with phosphate-buffered saline solution (PBS, pH 7.2) to wash out the residual blood. Both ends of the cord were clamped and then infused with 100 mg/l collagenase (Sigma Chemical Co, St. Louis, MO) in Hanks' balanced salt solution (HBSS, pH 7.2). After 10 min incubation at room temperature, the solution containing the detached endothelial cells was flushed out with PBS. The cells were then centrifuged and suspended in RPMI 1640 (pH 7.2) sup-
plemented with 20% heat-inactivated fetal bovine serum (FBS, GIBCO, Grand Island, NY), 25 mg/l endothelial cells growth supplement (EGGS, Sigma Chemical Co), 5×10^5 U/l heparin, and antibiotics (1×10^5 U/l penicillin and 100 mg/l streptomycin), and then cultured in culture dishes (Falcon 3000, Becton Dickinson Co, St. Louis, MO), precoated with 15 mg/l fibronectin (Sigma Chemical Co). Endothelial cells were identified by their characteristic morphology, such as a cobblestone appearance under phase-contrast microscopy, and by the enzyme-labelled antibody technique using a mouse monoclonal anti-human Von Willebrand Factor (Immunotech, Marseille, France). The endothelial cells preparation obtained by this method was more than 99% reactive with this antibody as determined by an immunohistological method (avidin-biotin immunoperoxidase technique). The endothelial cells at the 3rd or 4th passage were used for the following experiments.

**Preparation of thyrocytes**

Normal thyroid glands were obtained from surgical specimens adjacent to thyroid follicular adenomas. Thyroid tissue was also obtained from 5 patients with Graves' disease who were euthyroid at the time of subtotal thyroidectomy. Methods for thyrocyte preparation were previously described in detail (11,12). Thyroid tissues were digested with collagenase and dispase (Godo Shusei Co, Tokyo, Japan) in HBSS (pH 7.2). To eliminate non-adherent cells from thyrocyte preparations, the dispersed cells were cultured for 18 h in RPMI 1640 supplemented with 10% FBS and antibiotics, and then extensively washed with PBS containing 2% FBS. Adherent thyrocytes were removed from the culture dishes by adding trypsin-EDTA HBSS, and were further cultured in the RPMI 1640 medium containing 10% FBS. The thyrocyte preparations were less than 1% reactive with monoclonal antibodies, CD3 (Coulter Immunology, Hialeah, FL), Leu M3 (Becton Dickinson Co, Mountain View, CA), CD20 (Coulter Immunology, Hialeah, FL) and anti-human Von Willebrand factor, which define an antigen on all mature T cells, on monocyte/macrophages, on pan-B cells and on vascular endothelial cells, respectively. Moreover, thyrocyte preparations were more than 99% reactive with anti-thyroglobulin antibody by an immunohistological method (avidin-biotin immunoperoxidase technique).

**Proliferative responses of thyrocytes toward endothelial cells**

In order to determine whether endothelial cells were able to increase the proliferative responses of thyrocytes, cultures were performed in quadruplicate with fibronectin-coated 96-well flat bottomed microtitre plates (Costar, Cambridge, MA) that contained a mixture of 1×10^6 thyrocytes and 1×10^4 mitomycin C-treated endothelial cells in 200 μl of media consisting of RPMI 1640 supplemented with 10% FBS. Endothelial cells were treated with 50 mg/l mitomycin-C for 30 min. In some experiments, varying concentrations of heparin or indomethacin (Sigma Chemical Co) were added to the mixture of thyrocytes and endothelial cells. Twenty-four hours before termination of the culture, 0.15 μCi of [3H]thymidine (New England Nuclear, Boston, MA) was added to each well. The cells were harvested on glass filters using a semiautomatic cell harvester (Labo Mash, Labo Science, Tokyo, Japan), and the radioactivity of each sample was determined in a liquid scintillation counter. To examine the effect of fibroblast on thyrocyte proliferation, 1×10^4 thyrocytes were in some experiments cultured with or without 1×10^6 mitomycin C-treated human foreskin fibroblasts in 200 μl of RPMI 1640 supplemented with 10% FBS. Human foreskin fibroblasts were treated with 100 mg/l mitomycin-C for 60 min. Twenty-four hours before termination of the culture, 0.15 μCi of [3H]thymidine was added to each well and the radioactivity of each sample was determined in a liquid scintillation counter. [3H]thymidine incorporation of thyrocytes in co-culture experiments was calculated as follows: cpm of co-culture experiments – cpm of either endothelial cells of fibroblasts only. [3H]thymidine incorporation of either mitomycin C-treated endothelial cells or mitomycin C-treated fibroblasts was less than 40 cpm in all experiments. For an evaluation of the effect of growth-promoting factors on various types of cells, the method of [3H]thymidine incorporation is biased by many artefacts and the evaluation of increase in cell number by cell counting or DNA content is thought to be best (13). Therefore, we counted the cell number of thyrocytes. In co-culture experiment, the cell number of thyrocytes was calculated as follows: total cell number of co-culture experiment – cell number of mitomycin-C treated endothelial cells or mitomycin-C treated fibroblasts.

**Data analysis**

Student's t-test was used to determine the statistical significance. A p-value <0.05 was considered significant.

**Results**

Endothelial cells enhanced the proliferative responses of human thyrocytes. Thyrocytes from normal thyroid tissues were cultured in the presence or absence of mitomycin C-treated endothelial cells for varying periods. Fig. 1 shows a representative kinetic study of the co-culture experiments. The proliferative response of thyrocytes toward endothelial cells was significantly higher than that of thyrocytes at day 7, and reached a plateau at culture day 11 (stimulation index=3.1). The proliferative response of thyrocytes from patients with Graves' disease was similar to that of normal thyrocytes (data not shown). To determine whether the increased proliferative responses of
human thyrocytes were induced by endothelial cells, thyrocytes were cultured with or without mitomycin C-treated human foreskin fibroblasts. As shown in Fig. 2, fibroblasts did not stimulate thyrocyte proliferation. Next, we counted the cell number of thyrocytes as described in Materials and Methods. In the endothelial cell-thyrocyte co-culture experiment, the cell number of thyrocytes at culture day 11 was markedly increased as compared with that in the experiment on thyrocytes alone (thyrocytes alone 100±6%, co-culture 127±8%, p<0.01; cell number of thyrocytes in the experiment of thyrocytes alone was defined as 100%). In contrast to the endothelial cell-thyrocyte co-culture experiment, cell number of thyrocytes at culture day 11 in the fibroblast-thyrocyte co-culture experiment was not increased as compared with that in the experiment on thyrocytes alone (thyrocytes alone 100±8%, co-culture 96±10%, cell number of thyrocytes in the experiment of thyrocytes alone

Fig. 1.
Proliferative responses of human thyrocytes stimulated by endothelial cells. Normal thyrocytes were cultured in the presence or absence of mitomycin C-treated endothelial cells for varying periods. Twenty-four hours before termination of the culture, [³H]thymidine (³H-TdR) was added to each well and radioactivity in each sample was determined in a liquid scintillation counter. O—O: [³H]thymidine incorporation into unstimulated thyrocytes; •—•: [³H]thymidine incorporation into thyrocytes stimulated by endothelial cells. The results were the mean ± SD of quadruplicate cultures. *p<0.01, [³H]thymidine incorporation into unstimulated thyrocytes vs [³H]thymidine incorporation stimulated by endothelial cells.

Fig. 2.
Proliferative responses of thyrocytes stimulated by human skin fibroblasts. Thyrocytes were cultured with or without mitomycin C-treated fibroblasts for varying periods. O—O shows the proliferative responses of unstimulated thyrocytes. •—• shows the proliferative responses of thyrocytes stimulated by fibroblasts. The results are the mean ± SD of quadruplicate cultures.
was defined as 100%). Cell number of mitomycin-C treated endothelial cells or mitomycin-C treated fibroblasts at culture day 11 was not increased as compared with those at the start of the cultures. These results suggest that the increased proliferative response of thyrocytes may be attributed to endothelial cells and the increased $[^3]H$thymidine incorporation into thyrocytes might be responsible for thyrocyte proliferation. In addition, to clarify whether the endothelial cell-stimulated thyrocyte proliferation could be attributed to growth factors produced by endothelial cells or to direct contact between thyrocytes and endothelial cells, we added the culture medium of endothelial cells to thyrocytes. Endothelial cells ($1 \times 10^5/200 \mu l$ of culture medium) were cultured with RPMI 1640 supplemented with 10% FBS for 3 days. At the termination of culture, the culture medium was collected. Thyrocytes ($1 \times 10^5/well$) were cultured with the culture medium of endothelial cells for 11 days and the proliferative response was determined by $[^3]H$thymidine incorporation. The proliferative effect of culture medium was significant, but smaller than that in the co-culture experiment (endothelial cells medium 6050±420 cpm, co-culture 8852±521 cpm, thyrocytes alone 4170±303 cpm).

Effect of indomethacin and heparin on the increased proliferative response of thyrocytes induced by endothelial cells

Previously we have reported that interleukin 1 (IL-1) stimulated $[^3]H$thymidine incorporation into thyrocytes from normal subjects and patients with Graves’ disease (6). It was reported that fibroblast growth factor (FGF) acted upon mitogenic factors on thyrocytes (2). It became apparent that endothelial cells were capable of producing IL-1 (14) and basic FGF (15,16). An experiment was therefore undertaken to determine whether the increased proliferation of thyrocytes co-cultured with endothelial cells was dependent on either IL-1 or basic FGF produced by endothelial cells. Previously we have reported that the cyclooxygenase inhibitor, indomethacin, completely inhibits the rIL-1-stimulated prostaglandin E$_2$ production and markedly increases $[^3]H$thymidine incorporation into thyrocytes (6). Varying amounts of indomethacin were added to the mixture of thyrocytes and endothelial cells. As shown in Fig. 3, indomethacin did not increase $[^3]H$thymidine incorporation into thyrocytes. Because the proliferative effect of basic FGF on endothelial cells was reported to be suppressed by heparin (17), the mixtures of thyrocytes and endothelial cells were cultured with or without varying concentrations of heparin. As shown in Fig. 4, heparin did not inhibit the endothelial cells-stimulated thyrocyte proliferation. From the above results, it seems unlikely that IL-1 or FGF may play an important role in the increased proliferative response of thyrocytes induced by endothelial cells.

Contamination of mononuclear cells in endothelial cell-thyrocyte co-culture experiments

Endothelial cells have been shown to stimulate lymphocyte proliferation (18). It is possible that the increase in $[^3]H$thymidine incorporation into the mixtures of thyrocytes and endothelial cells depends on the proliferative responses of contaminated mononuclear cells in these experiments. As described in Materials and Methods, the preparations of thyrocytes and endothelial cells were less than 1% reactive with the monoclonal antibodies, CD3, CD8, and CD19.

**Fig. 3.** Effects of indomethacin on the proliferative responses of thyrocytes stimulated by endothelial cells. The mixtures of thyrocytes and mitomycin C-treated endothelial cells were cultured with varying amounts of indomethacin for 11 days. The results are mean ± SD in quadruplicate cultures.
Fig. 4.
Effects of heparin on the proliferative responses of thyrocytes stimulated by endothelial cells. Varying concentrations of heparin were added to the mixtures of thyrocytes and mitomycin C-treated endothelial cells. The mixtures were cultured for 11 days. The results are the mean ± SD in quadruplicate cultures.

Leu M3 and CD20, respectively. We also examined the phenotypic characterization of the cell preparations at day 11 of co-culture using a flow cytometer (EPICS-C cell sorter, Coulter Electronics, Hialeah, FL). As shown in Table 1, few cells were present in the region of lymphocytes. Less than 1% of the cells in the region of thyrocytes were stained with the monoclonal antibodies, CD3, Leu M3 and CD20. From the above findings, we concluded that the increased [3H]thymidine incorporation was responsible for thyrocyte proliferation.

Table 1.
Cell components of endothelial cell-thyrocyte co-culture experiments.

<table>
<thead>
<tr>
<th>Region</th>
<th>Monoclonal antibodies</th>
<th>% of positive cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymphocyte</td>
<td>anti-CD3</td>
<td>non-detectable</td>
</tr>
<tr>
<td>region</td>
<td>anti-Leu M3</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thyrocyte</td>
<td>anti-CD3</td>
<td>0.9%</td>
</tr>
<tr>
<td>region</td>
<td>anti-CD20</td>
<td>0.5%</td>
</tr>
<tr>
<td></td>
<td>anti-Leu M3</td>
<td>0.9%</td>
</tr>
</tbody>
</table>

Phenotypic characterization of 11 days co-cultured cells. Two regions, either lymphocyte or thyrocyte regions, were determined and cell surface markers were determined by fluorocytometric analysis using an EPICS-C cell sorter.

Discussion
The present study demonstrated that endothelial cells could stimulate proliferation of thyrocytes obtained from normal thyroid tissue thyroid tissue from and patients with Graves' disease. Endothelial cells were previously shown to stimulate lymphocyte proliferation (18). Our mixture of thyrocytes and endothelial cells were not contaminated with T cells, B cells or monocytes/macrophages. The increased [3H]thymidine incorporation in the mixture of thyrocytes and endothelial cells may be attributed to the proliferation of thyrocytes because cell number of thyrocytes increased in the endothelial cell-thyrocyte co-culture experiment.

IL-1 is a potent inducer of prostaglandin synthesis in a variety of cells, including fibroblasts (19), chondrocytes (20), monocytes (21), vascular endothelial cells (22), vascular smooth muscle cells (19), and human thyrocytes (6). Endogenous or exogenous prostaglandins inhibit cell proliferation (23). We have reported that indomethacin, a cyclooxygenase inhibitor, completely inhibited prostaglandin E2 production of thyrocytes in response to IL-1 and markedly increased [3H]thymidine incorporation into thyrocytes (6). In the present study, the stimulatory effects of endothelial cells on human thyrocytes was not enhanced in the presence of indomethacin in the endothelial cell-thyrocyte co-culture. These data support the idea that IL-1 may not play any role in the stimulatory effect of endothelial cells on thyrocyte proliferation.
It was reported that FGF is a mitogenic factor on thyrocytes (2). In the FGF family, two types of polypeptides exist as acidic FGF and basic FGF (17). Endothelial cells are able to produce basic FGF (14,15). The proliferative effect of basic FGF is suppressed by heparin (17). We found that the endothelial cells-stimulated increased [3H]thymidine incorporation into thyrocytes was not blocked by heparin. These results indicate that the stimulatory effect of endothelial cells on thyrocyte proliferation may not be attributed to FGF released by endothelial cells. Recently it became apparent that growth factors released by mechanically wounded endothelial cells (24) or irradiated endothelial cells (25) were partially defined as FGF. We could not exclude the possibility that mitomycin C-treated endothelial cells may release basic FGF, resulting in proliferation of human thyrocytes.

Platelet-derived growth factor (PDGF), which is a growth factor for vascular smooth muscle cells and fibroblasts, is known to be produced from endothelial cells (26). Fibroblasts are also able to produce PDGF (27). In our study, fibroblasts could not stimulate thyrocyte proliferation and to our knowledge, PDGF has not been reported to be a growth factor for thyrocytes. Therefore, it seems unlikely that PDGF produced by endothelial cells may act as a growth factor for thyrocytes. There are other humoral factors which possess growth-promoting activity for thyrocytes, such as EGF (1-3) and IGF-I (4,5). Production sites of EGF are widely distributed in the human body (28) and production of prostaglandin E2 in thyrocytes is increased by EGF (3). IGF-I is also reported to be produced from fibroblasts (29). Our study demonstrated that fibroblasts were unable to stimulate thyrocyte proliferation and the addition of indomethacin into the mixtures of thyrocytes and endothelial cells did not increase thyrocytes proliferation. Therefore, it appears that neither EGF or IGF-I may be responsible for the thyrocyte proliferation stimulated by endothelial cells.

We were unable to determine whether increased thyrocyte proliferation stimulated by endothelial cells could be attributed to growth factor(s) produced by endothelial cells or to direct contact between thyrocytes and endothelial cells. The culture medium of endothelial cells was also able to increase the thyrocyte proliferation. Endothelin, which is reported to be released from endothelial cells (30,31), may be responsible for the thyrocyte proliferation. However, the culture medium of endothelial cells only partly had any effect in the endothelial cell-thyrocyte co-culture experiment, indicating that cell contact or extracellular matrix production by endothelial cells may play a critical role in the proliferation of thyrocytes. Nevertheless, there are no reports that endothelial cells can stimulate thyrocyte proliferation. Numerous small blood vessels appeared to be actively proliferating in thyroid tissues from patients with Graves' disease and Hashimoto's thyroiditis. Proliferation of small blood vessels is essentially dependent on the local proliferation of endothelial cells (7). Furthermore, the proliferation of endothelial cells precedes that of thyrocytes in experimental models of thyroid hyperplasia (8). Our present results suggest that endothelial cells may regulate thyrocyte proliferation and contribute to the development of thyroid enlargement in patients with autoimmune thyroid disease.

References


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