Stimulation of mitogenesis in human thyroid epithelial cells by endothelin

Katsumi Eguchi, Atsushi Kawakami, Munetoshi Nakashima, Hiroaki Ida, Souko Sakito, Masahiro Sakai, Kaoru Terada, Yojiro Kawabe, Takaaki Fukuda, Naofumi Ishikawa, Kunihiko Ito and Shigenobu Nagataki

First Department of Internal Medicine, Nagasaki University School of Medicine, Nagasaki, and Ito Hospital, Tokyo, Japan

We investigated whether a potent vasoconstrictor, endothelin, stimulated the proliferation of human thyroid epithelial cells (thyrocytes). [3H]-thymidine incorporation into normal thyrocytes and thyrocytes from patients with Graves’ disease was significantly increased at 10^{-7} \text{ mol/l} endothelin, reaching a plateau at 10^{-6} \text{ mol/l}. The proliferative responses of the thyrocytes obtained from patients with Graves’ disease were similar to those of normal thyrocytes. Furthermore, the cell number of thyrocytes stimulated by endothelin was increased as compared with that of unstimulated thyrocytes. Neither indomethacin nor heparin affected this endothelin-stimulated thyrocyte proliferation. When thyrocytes were cultured with both endothelin and recombinant interleukin 1β, there was an additive effect on thyrocyte proliferation. The Ca^{2+} entry blocker, verapamil, inhibited both the proliferative responses of thyrocytes to endothelin and the additive effect of endothelin and recombinant interleukin 1β on thyrocyte proliferation. These results suggest that endothelin functions as a growth-promoting factor for human thyrocytes, presumably through intracellular calcium influx.

Katsumi Eguchi, First Department of Internal Medicine, Nagasaki University School of Medicine, 7-1 Sakamoto-machi, Nagasaki 852, Japan

Thyroid tissues from patients with Graves’ disease are characterized by pronounced hyperplasia of their thyroid epithelial cells, the generation of new blood vessels and infiltration by mononuclear cells. The neovascularization is shown to depend on the local proliferation of vascular endothelial cells (ECs). In an experimental model of thyroid hyperplasia, proliferation of ECs in the small blood vessels preceded hyperplasia of the thyroid follicular cells (1). Recently we reported that human umbilical vein ECs stimulated the proliferation of human thyroid epithelial cells (2). The ECs release paracrine factors that include eicosanoids, cyclic nucleotides, endothelium-derived relaxing factor and angiotensin II, which regulate the vasoreactivity and metabolism of the vascular smooth muscle cells (3–6). A potent vasoconstrictor peptide, endothelin, has been reported to be released by porcine and human ECs (7, 8). In addition, endothelin-induced vasoconstriction has been shown to be mediated by Ca^{2+} entry via plasma membrane Ca^{2+} channels (7).

We tested whether endothelin stimulates the proliferation of thyroid epithelial cells (thyrocytes), because many constrictor peptides activate Ca^{2+} signalling through phospholipase C and induce mitogenesis (9–11).

Materials and methods

Preparation of thyrocytes

Normal thyroid tissues adjacent to thyroid follicular adenomas were taken from surgical specimens. Thyroid tissues were also obtained from Graves’ disease patients who were euthyroid at the time of subtotal thyroidectomy. The methods used to prepare the thyrocytes have been reported elsewhere in detail (12, 13). In brief, the thyroid tissues were digested with collagenase (Sigma Co, St Louis, MO) and dispase (Godo Shusui Co, Tokyo, Japan) in Hank’s balanced salt solution (HBSS). To eliminate non-adherent cells from the thyrocyte preparations, the dispersed cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS, Gibco, Grand Island, NY) on culture dishes (Falcon 3003, Becton Dickinson Co., Oxnard, CA) for 18 h then washed extensively with phosphate-buffered saline (PBS) containing 2% fetal bovine serum (FBS, Gibco, Grand Island, NY). The adherent thyrocytes were removed from the culture dishes by adding trypsin-EDTA-HBSS and washing them three times in PBS containing 2% FBS. The thyrocytes obtained were cultured in culture dishes with RPMI1640 supplemented with 10% FBS and the antibiotics (1 × 10^{3}U/l penicillin and 100 mg/l...
streptomycin). The thyrocyte preparations obtained were less than 1% reactive with the monoclonal antibodies CD3 (Coulter Immunology, Hialah, FL), LeuM3 (Becton Dickinson Co, Mountain View, CA), CD20 (Coulter Immunology, Hialah, FL) and Von Willebrand factor (Immunotech, Marseilles, France), antibodies which respectively define an antigen on all mature peripheral blood T cells, on monocytes/macrophages, pan B cells, and on vascular endothelial cells. Moreover, an immunohistological method (avidin-biotin immunoperoxidase technique) showed that the thyrocyte preparations were more than 99% reactive with antithyroglobulin antibody.

**Thyrocyte proliferation assay**

Thyrocytes (1 x 10^4/well) from normal thyroid tissue or thyroid tissue from patients with Graves’ disease were cultured for 24 h in 200 μl of culture medium in quadruplicate in 96-well, flat-bottomed microtiter plates (Costar, Cambridge, MA). The following day, RPMI 1640 supplemented with 5% FBS and various concentrations of reagents were added to each well and culture was continued. Human endothelin-1, which was purchased from Peptide Institute, Inc, Osaka, Japan was synthetic peptide (MW 2491.9). The contamination of the preparation was less than 1%. The reagents used in these experiments were recombinant IL-1β (rIL-1β) (Otsuka Pharmaceutical Co, Tokushima, Japan); indomethacin (Sigma Co, St Louis, MO) and heparin (Sigma Co, St Louis, MO). Twenty-four hours before the termination of culture. 0.15 μCi of [3H]-thymidine (NEN, 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), after which radioactivity of each sample was determined with a liquid scintillation counter. Furthermore, the cell numbers of thyrocytes were counted using the trypan blue dye exclusion test.

**Data analysis**

Student’s t-test was used to determine the statistical significance. P values of <0.05 were considered significant.

**Results**

**Effect of endothelin on the proliferative response of human thyroid epithelial cells**

To determine whether endothelin stimulated the proliferation of thyrocytes, we added endothelin (10^-8 mol/l) to cultures of normal thyrocytes then incubated them for various periods. As shown in Fig. 1, the proliferative response of the thyrocytes was significantly increased by an addition of endothelin in comparison to the response of thyrocytes alone, reaching a peak on day 7.

We next studied the proliferative response of thyrocytes cultured with various amounts of endothelin. [3H]-thymidine incorporation in the thyrocytes was significantly increased at 10^-9 mol/l endothelin, reaching a...
plateau at $10^{-8}$ mol/l (Fig. 2). In subsequent experiments, therefore, the proliferative responses of the thyrocytes were determined on day 7 of culture and at a concentration of $10^{-8}$ mol/l endothelin. The proliferative response of thyrocytes from patients with Graves’ disease toward endothelin was similar to that of normal thyrocytes (data not shown). Therefore, normal thyrocytes were used in the following experiments. Next, we counted the cell number of thyrocytes cultured with or without $10^{-8}$ mol/l endothelin. Compared to unstimulated thyrocytes (defined as 100%), the number of endothelin-stimulated thyrocytes was increased (134 ± 7%).

**Effects of indomethacin or heparin on the proliferative response of thyrocytes stimulated with endothelin**

Previously we demonstrated that IL-1β-stimulated [3H]-thymidine incorporation into thyrocytes and production of prostaglandin E2 (14). Indomethacin, cyclooxygenase inhibitor, completely inhibited IL-1β-stimulated prostaglandin E2 production but increased [3H]-thymidine incorporation markedly (14). Therefore, we ascertained whether an addition of indomethacin to thyrocyte cultures enhanced the thyrocyte proliferation response to endothelin. In the absence of indomethacin, endothelin-stimulated thyrocyte [3H]-thymidine uptake, and when thyrocytes were cultured with endothelin in the

---

**Fig. 3.** A. Effect of indomethacin on endothelin-induced thyrocyte proliferation. Thyrocytes were cultured for seven days in various concentrations of indomethacin in the presence of $10^{-8}$ mol/l endothelin. [3H]-thymidine incorporation into the thyrocytes was determined as described in Materials and Methods. C: control, cultured in the absence of endothelin and indomethacin. Indomethacin up to 5.0 mg/l had no effect on proliferation of thyrocytes. Three experiments were carried out with similar results. The figure is a representative experiment. The bars are mean ± S.D. (N = 4). *p < 0.01, vs control. B. Effect of heparin on endothelin-induced thyrocyte proliferation. Thyrocytes were cultured for seven days in various concentrations of heparin in the presence of $10^{-8}$ endothelin. C: control, cultured in the absence of endothelin and heparin. Heparin had no effect on proliferation of thyrocytes. Three experiments were carried out with similar results. The figure is a representative experiment. The bars are mean ± S.D. (N = 4). *p < 0.01, vs control.

**Fig. 4.** Additive effect of endothelin together with IL-1β on thyrocyte proliferation. Thyrocytes were cultured for seven days in the presence or absence of various reagents: $10^{-8}$ mol/l endothelin, $1.0 \times 10^4$ IU/l rIL-1β, and 1.0 mg/l indomethacin. [3H]-thymidine incorporation was determined as described in Materials and Methods. Three experiments were carried out with similar results. The figure is a representative experiment. The bars are mean ± S.D. (N = 4). *p < 0.01, vs unstimulated thyrocytes. **p < 0.01, vs thyrocytes cultured in $10^{-8}$ mol/l endothelin.
Dose response relationship of IL-1β on endothelin-induced thyrocyte proliferation. Thyrocytes were cultured for seven days in various concentrations of rIL-1β in the presence of 10^{-8} mol/l endothelin. Four experiments were carried out and similar data were obtained. The figure is a representative experiment. The bars are mean ± sd (N = 4). *p < 0.05, vs thyrocytes cultured in 10^{-8} mol/l endothelin. ** p < 0.01, vs thyrocytes cultured in 10^{-8} mol/l endothelin.

Additive effect of endothelin combined with IL-1β on thyrocyte proliferation

We investigated the combined effect of endothelin and IL-1β on thyrocyte proliferation. Normal thyrocytes were cultured for seven days with 10^{-8} mol/l endothelin in the presence or absence of 1.0 × 10^5 IU/l rIL-1β. Under these experimental conditions, rIL-1β alone slightly increased [³H]-thymidine incorporation into thyrocytes; but when indomethacin was added to the cultures as well, [³H]-thymidine incorporation increased (Fig. 4). Endothelin alone significantly increased the thyrocyte proliferation. Moreover, when thyrocytes were cultured with both rIL-1β and endothelin, the cytokines had an additive effect on thyrocyte proliferation (Fig. 4).

presence of indomethacin, the proliferative response of the thyrocytes was not increased (Fig. 3A). Thus, the addition of indomethacin to thyrocyte cultures did not enhance the thyrocyte proliferative response to endothelin. The proliferative response of the thyrocytes was unaffected by the addition of heparin (15). Heparin enhancing the growth-promoting activity of acidic FGF but inhibiting that of basic FGF (15). To determine whether heparin affected the endothelin-induced thyrocyte proliferation, we cultured normal thyrocytes with various amounts of heparin in the presence of 10^{-8} mol/l endothelin. Heparin did not affect endothelin-induced thyrocyte proliferation (Fig. 3B).
Effect of indomethacin on the additive proliferative response of thyrocytes stimulated with rIL-1β and endothelin

Indomethacin stimulated rIL-1β-induced thyrocyte proliferation, but had no effect on endothelin-induced thyrocyte proliferation. We therefore investigated the effect of indomethacin on the additive proliferative response of thyrocytes stimulated by rIL-1β and endothelin together. Thyrocytes were cultured for seven days with various concentrations of indomethacin in the presence of 10^{-8} mol/l endothelin and 1.0 IU/ml rIL-1β. Indomethacin could not increase the [3H]-thymidine incorporation into thyrocytes stimulated by the two cytokines (Fig. 6).

Effect of a calcium entry blocker on endothelin-stimulated thyrocyte proliferation

The vasoconstriction induced by endothelin has been shown to be inhibited by the addition of a calcium entry blocker (5). To determine whether endothelin-induced thyrocyte proliferation was suppressed by a calcium entry blocker, we cultured thyrocytes with various concentrations of the calcium entry blocker verapamil in the presence of 10^{-8} mol/l endothelin. As shown in Table 1, verapamil inhibited endothelin-induced thyrocyte proliferation. Moreover, it inhibited the additive effect of endothelin and rIL-1β on thyrocyte proliferation. In the absence of endothelin, neither verapamil nor combinations of verapamil and IL-1β had any effect on the proliferations of thyrocytes (data not shown).

Discussion

Endothelin is known to be a potent vasoconstrictive peptide (7), and it has become apparent that it has a variety of functions, such as stimulation of atrial natriuretic peptide release from rat atria (16), inhibition of renin release (17) and stimulation of aldosterone biosynthesis (18). We have demonstrated here that endothelin stimulated the proliferation of thyroid epithelial cells from normal thyroid tissue and tissue from patients with Graves’ disease. Endothelin has now been reported to function as a mitogen in rat glomerular mesangial cells, but not in Swiss 3T3 fibroblasts (19). These findings suggest that the mitogenic effect of endothelin is not a ubiquitous response. We reported elsewhere that rIL-1β stimulated [3H]-thymidine incorporation into thyrocytes and increased the ratio of thyrocytes in the S phase of the cell cycle (14). Moreover, thyrocytes produced significant amounts of prostaglandin E_2 in response to rIL-1β (14). Because endogenous and exogenous prostaglandins inhibit cell proliferation (20), the cyclooxygenase inhibitor indomethacin is able to enhance rIL-1β-stimulated thyrocyte proliferation. These results suggest that the direct
stimulatory effect of IL-1 on human thyrocyte growth may be counterbalanced by an autocrine inhibitory loop involving thyrocyte PG synthesis (14). Our study has demonstrated that endothelin did not increase the proliferation of human thyrocytes in the presence of indomethacin. Therefore, the possibility that thyrocytes stimulated with endothelin produce prostaglandins is excluded.

Heparin has been used to characterize and separate growth-promoting factors. It potentiates the growth-promoting activity of acidic fibroblast growth factor (FGF), but inhibits the mitogenic effects of basic FGF (15). The present study has shown that heparin did not affect the endothelin-stimulated thyrocyte proliferation. These results suggest that endothelin-stimulated thyrocyte proliferation is not mediated by the production of basic and acidic FGF.

We have shown here that endothelin together with rIL-1β had an additive effect on the proliferation of human thyrocytes; whereas rIL-1β alone could slightly enhance thyrocyte proliferation. These results do not agree with previous findings that rIL-1β enhances thyrocyte proliferation (14). The discrepancy in these findings is probably due to different periods of cultures; the effects of IL-1β were investigated after four days, whereas the effects of endothelin were studied after seven days. To our knowledge, the additive effect of endothelin and rIL-1β on thyrocyte proliferation has not previously been reported. Endothelin has been shown to be a mitogen and to require either a co-factor in FBS, or the presence of a competence factor, such as insulin, to express mitogenic activity in mesangial cells (19). The mesangial cell proliferation induced by endothelin has been reported to be accompanied by the activation of phospholipase C with increased inositol phosphate turnover and increments of intracellular [Ca2+] (19). We have shown here that verapamil, a calcium entry blocker inhibited endothelin-induced thyrocyte proliferation and also inhibited the additive effect of endothelin and rIL-1β on thyrocyte proliferation, which suggests that endothelin-induced thyrocyte proliferation is mediated by an intracellular [Ca2+] influx. The actual mechanism by which endothelin affects thyrocyte proliferation, however, has yet to be clarified.

In summary, our results indicate that endothelin stimulates thyrocyte proliferation and that the local production of endothelin by ECs of new blood vessels may contribute to the thyrocyte proliferation found in patients with Graves’ disease. Moreover, the IL-1 produced by infiltrating mononuclear cells in combination with endothelin may also contribute to the proliferation of thyrocytes.

Acknowledgments. We are grateful to Miss M Matsuo for her excellent experimental assistance, and to Miss C Tsuruta and Y Takahara for excellent secretarial assistance.

References

Received March 11th, 1992
Accepted October 2nd 1992