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Production of thymocyte-stimulating activity by cultured human thyroid epithelial cells

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SUMMARY

Since thyroid follicular epithelial cells (thyrocytes) have been shown to express a number of functions similar to monocytes, they were further examined for their potency in secreting thymocyte-stimulating activity (TSA). Although spontaneous production of TSA could not be detected when thyrocytes were cultured in the culture medium, TSA was demonstrated in the culture supernatants after stimulation with the immune adjuvant lentinan. The release of TSA was found in the culture supernatants collected 24 h after stimulation and was maintained for the 4 days of culture. Maximum levels of TSA release were achieved by 2 days. In addition, when culture supernatants of thyrocytes stimulated with lentinan or monocyte-derived interleukin 1 (IL-1) were incubated with a rabbit antibody to human IL-1, a parallel reduction in TSA was observed, suggesting that the active product in the thyrocyte culture supernatant shared a common antigenic site with IL-1. The demonstration of the production of IL-1 like activity by thyrocytes provides additional evidence that these cells, in addition to their functions as endocrine cells, may also participate in the local immune responses under appropriate conditions.

Keywords autoimmune thyroid disease thyrocyte interleukin 1 thymocyte-stimulating activity

INTRODUCTION

In autoimmune thyroid disease, a variety of hypotheses about the mechanisms underlying autoimmunity have been proposed (Bottazzo, Pujol-Borrell & Hanafusa, 1983; Jansson, Karlsson & Forsum, 1984; Davies, 1985). Pujol-Borrell and coworkers (1983) demonstrated that thyrocytes, which were normally negative for the class II major histocompatibility complex (HLA-DR), expressed class II antigens after lectin stimulation, and it has been also found that thyrocytes from patients with autoimmune thyroid disease are positive for HLA-DR *in vivo* (Hanafusa *et al.*, 1983). The HLA-DR positive thyrocytes have been considered to act as immuno-competent cells and to play an important role in the development of autoimmune thyroid disease by interacting with T cells (Bottazzo *et al.*, 1983; Davies, 1985).

It is well established that T cells recognize antigens in association with HLA-DR found on the surface of antigen-presenting cells and are activated by IL-1 secreted from the same cells (Berle & Thorsby, 1980; Smith *et al.*, 1980; Mizel, 1982). Also it has been hypothesized that both IL-1 and HLA-DR (or Ia) are essential for accessory cell function (Oppenheim *et al*, 1984). IL-1 activity is produced not only by monocytes/macrophages, but by many other types of cells such as

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keratinocytes (Luger *et al.*, 1981), mesangial cells (Lovett, Ryan & Sterzel, 1983), endothelial cells (Miossec, Cavender & Ziff, 1985) and Epstein–Barr virus transformed human B cells (Chu *et al.*, 1985). If thyrocytes are capable of impacting T cells, their secretion of IL-1 might be expected.

In this study, we demonstrate HLA-DR expression and the production of IL-1-like activity by cultured normal human thyrocytes after stimulation with lentinan, an anti-tumour polysaccharide.

MATERIALS AND METHODS

Medium. Dulbecco's Modification of Eagle's Medium (MEM) (Flow Laboratories, McLean, Virginia) or RPMI 1640 (GIBCO Laboratories, Grand Island NY) were supplemented with 100 IU/ml penicillin and 100 μg/ml streptomycin. Heat-inactivated fetal calf serum (FCS) (GIBCO Laboratories) was added as indicated.

Culture and identification of human thyrocytes. Normal human thyroid tissue was obtained from around benign adenoma at surgery. The thyrocytes were prepared and cultured as described elsewhere (Khoury *et al.*, 1981). By light microscopic findings, the majority of cells in early thyroid monolayer cultures were thyrocytes. They were further identified by indirect immunofluorescence (IF) staining with microsomal antibody-positive sera (as described by Khoury *et al.*, 1981). The cell preparations contained more than 95% thyrocytes.

Identification of other cell types in primary thyroid cell cultures. Five-day-old culture preparations were tested for the identification of the other cell types. Macrophages were identified by IF staining with monoclonal anti-HLA-DR antibody (OKIal, Ortho Pharmaceutical Co., Raritan, NJ) and with OKM1 (Ortho Pharmaceutical Co.), and non-specific esterase staining (Mutoh Pharmaceutical Co., Tokyo). The culture preparations contained less than 0·5% macrophages. IF staining of the cell cultures was negative for anti-factor VIII antibody (Cappel Laboratories, Inc, Cochranville, Pa). T cell and B cell contaminations were identified by IF staining with OKT3 (Ortho Pharmaceutical Co.) and anti-human immunoglobulin (Cappel Laboratories), respectively. The culture preparations contained less than 0·5% T cells and less than 0·5% B cells. Fibroblast contamination was evaluated by a leucine aminopeptidase staining (Jacquemont & Purniera, 1969), and never exceeded 1% of total cell population.

Generation of thymocyte stimulating activity (TSA) by thyrocytes. For the generation of TSA, 5-day-old monolayer cultures of normal human thyrocytes were employed. The cell preparations were stimulated with 0–10 μg/ml lentinan (Ajinomoto Co., Tokyo) in the presence of 5 μg/ml indomethacin for 12 h. After stimulation, the culture supernatants were harvested and replaced with fresh culture medium without lentinan every day. The daily collected samples were stored frozen at −20°C. Control supernatants were obtained from normal human thyroid monolayer cultures without lentinan stimulation in the same manner.

Elimination of monocytes with OKM1. Suspensions of thyrocytes were treated with OKM1 plus rabbit complement. After this treatment, the residual thyrocytes were cultured and examined for their production of TSA on stimulation with lentinan as described above.

Effect of lentinan on HLA-DR expression in cultured normal thyrocytes. Effect of lentinan (1·0 μg/ml) on HLA-DR expression in thyroid monolayer cultures was examined before and after stimulation. After IF staining with anti-HLA-DR monoclonal antibody, the number of cells positive for antigen per 100 cells in three different fields per culture preparation were counted under a fluoromicroscope. The results were expressed as the mean percent of triplicate cultures.

Preparation of standard IL-1 supernatant. Monocytes were obtained from peripheral blood mononuclear cells of a healthy donor by adhesion to FCS-coated Petri dishes (100 × 15 mm; Becton Dickinson Labware, Oxnard, CA) as described by Kurnick *et al.* (1979). The adherent cells were suspended at 10^6 cells/ml in RPMI 1640 with 10% FCS and were then stimulated with 0–10 μg/ml lentinan for 12 h. After stimulation, the culture supernatants were harvested and stored as described in the preceding section.

Blocking studies. Rabbit antibody to human IL-1 (anti-IL-1 antibody) was obtained from the Genzyme Corp., Boston, MA. This anti-IL-1 antibody was incubated at a final concentration of 1%

Table 1. Effect of lentinan and PHA on Balb/c mice thymocyte proliferation

Stimulant		ct/min ^{3}H-TdR incorporation
PHA (1·0 μg/ml)	Lentinan (μg/ml)	(mean ± s.d.)*
−	−	521 ± 12
+	−	480 ± 80
−	0·01	416 ± 101
−	0·1	376 ± 79
−	1·0	453 ± 116
−	10	440 ± 70
+	0·01	486 ± 51
+	0·1	443 ± 24
+	1·0	474 ± 98
+	10	492 ± 81

The direct effect of various concentrations of lentinan (0·01–10 μg/ml) and its synergistic effect with PHA on Balb/c mice thymocyte proliferation were tested.

* Mean ct/min ± s.d. of triplicate cultures.

with TSA or with standard IL-1 for 3 h at room temperature. Samples before and after such treatment were assayed for IL-1 activity.

Thymocyte proliferation assay. Thymocytes were prepared from 6 to 8-week-old Balb/c mice. Thymocytes were suspended in MEM supplemented with 10% FCS and $2{\cdot}5 \times 10^{-5}$M 2-mercaptoethanol and plated in 96-well, round-bottomed microtitre plates (Coster, Cambridge, MA) at a concentration of 10^6 cells/well in the presence or absence of 1·0 μg/ml phytohaemagglutinin (PHA) (Wellcome Reagents Limited, Beckman). Various dilutions of TSA containing supernatants of thyrocyte cultures, standard IL-1 or various concentrations of lentinan were added in triplicate. After 3 days of culture, the wells were pulsed with 0·4 μCi of methyl-(^{3}H) thymidine (^{3}H-TdR). The cells were automatically harvested 16 h later by cell harvester, and radioactivity counted in a liquid scintillation counter. Results were expressed as the mean ct/min ± s.d. of triplicate cultures or as a stimulation index (SI) compared with counts of the culture without lentinan-stimulation.

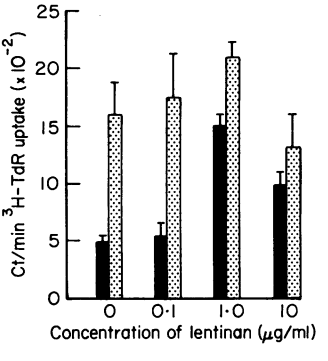

Fig. 1. 5-day-old thyrocytes (■) and peripheral blood monocytes (□) were stimulated with 0·1–10 μg/ml lentinan for 12 h. After the stimulation, the cells were washed with culture medium and were incubated in fresh medium for a further 24 h. The culture supernatants were then collected, and TSA was assayed on thymocyte proliferation. Results are expressed as the mean ct/min ± s.d. of triplicate cultures. Thymocytes incubated with 1·0 μg/ml PHA alone incorporated 440 ± 49 ct/min.

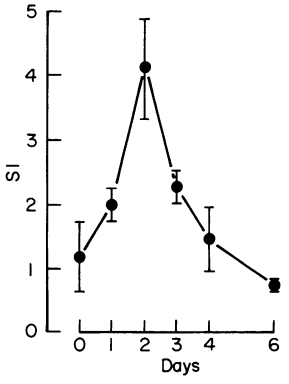

Fig. 2. 5-day-old thyrocytes ($n=5$) were stimulated with or without 1·0 μg/ml lentinan for 12 h. After the stimulation, the culture supernatants were collected and were replaced with fresh medium every day. TSA in these samples were assayed on thymocyte proliferation. Results are expressed as stimulation index (SI) as follows:

$$SI = \frac{\text{ct/min with lentinan-stimulation}}{\text{ct/min without lentinan-stimulation}}$$

RESULTS

Effect of lentinan on Balb/c mice thymocytes. Since thyrocytes or monocytes were stimulated with lentinan to produce TSA (or IL-1) in the present study, there is a possibility that the residual lentinan in the culture supernatants can influence the Balb/c mice thymocyte proliferation used to detect IL-1 activities. Therefore, it was necessary to test the direct effect of lentinan and the possible syngergistic effect of the agent and PHA on thymocyte proliferation. Balb/c mice thymocytes were directly stimulated with various concentration of lentinan (0·01–10 μg/ml) in the presence or the absence of PHA. As shown in Table 1, lentinan itself did not augment the thymocyte proliferation. Furthermore, synergistic thymocyte proliferative activities of lentinan and PHA were not found.

Optimal concentration of lentinan for the production of TSA. Lentinan was tested on cultured human normal thyrocytes at concentrations of 0·1–10 μg/ml. TSA in the culture supernatants

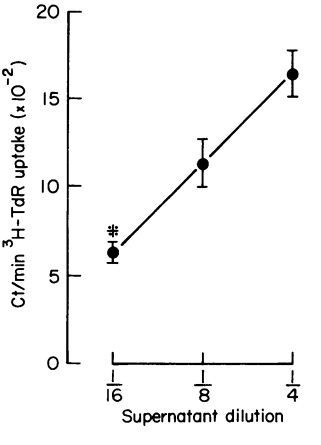

Fig. 3. Dependence of thymocyte proliferation on the concentration of TSA from thyrocyte cultures was observed ($r=0·96$, $P<0·001$). Results are expressed as the mean ct/min ± s.d. of triplicate cultures. Thymocytes incubated with 1·0 μg/ml PHA alone incorporated 412 ± 28. * Values significantly greater than those obtained from thymocyte cultures with PHA alone. $P<0·05$.

Table 2. Effect of anti-IL-1 antibody on TSA derived from macrophages and thyrocytes

Cell sources	^{3}H-TdR incorporation		Percent decrease*
	Medium	Ani-IL-1	
—	521±101	536±22	—
Macrophages	2181±203	1036±37	69
Thyrocytes	1606±176	1041±156	51

Culture supernatants of lentinan-stimulated thyrocytes and standard IL-1 were treated with rabbit antibody to human IL-1. TSA in both of these samples was down to the same levels by this treatment.

* % Decrease = ct/min in thyrocyte or macrophage cultures with anti-IL-1 − ct/min in medium/ct/min in thyrocyte or macrophage cultures − ct/min in medium.

Results are expressed as the mean ct/min ± s.d. of triplicate cultures.

collected 24 h after stimulation with lentinan were assayed on thymocyte proliferation. The effect of the agent on peripheral blood monocytes was also tested. These results are presented in Fig. 1. The optimal concentration of lentinan for TSA release by thyrocytes, as well as IL-1 release by monocytes, was 1·0 μg/ml. In the succeeding experiments, thyrocytes were stimulated with lentinan at this concentration. The levels of TSA produced by thyrocytes were much lower than that produced by monocytes. Although monocytes produced IL-1 spontaneously, TSA release by thyrocytes could not be detected without lentinan-stimulation.

Kinetics of TSA release by thyrocytes cultured with lentinan. As shown in Fig. 2, insignificant TSA was present in the culture supernatants removed soon after stimulation with lentinan (on day 0). However, release of TSA was demonstrable in the culture supernatants collected 24 h after the stimulation and was maintained for 4 days of culture. Maximum levels of the release of TSA was achieved by 2 days. Therefore, in order to produce maxium amounts of TSA for further analyses, we harvested samples after 2 days. The degree of thymocyte stimulation was dependent on the concentration of TSA contained in the culture supernatants added to the assay. TSA was demonstrable down to a 1/16 dilution (Fig. 3).

Table 3. Effect of elimination of monocytes with OKM1 on the production of TSA

Exp.	Treatment of thyrocytes with OKM 1 antibody	TSA (ct/min ± s.d.)
1	−	4250±1012
	+	4020± 62
2	−	2760± 121
	+	3011± 280

Contaminating monocytes in freshly dispersed thyrocytes were eliminated by cytokilling with OKM1 and rabbit complement. The effect of such treatment on the subsequent production of TSA on the stimulation with lentinan was tested. Results are expressed as the mean ct/min ± s.d. of triplicate cultures.

Effect of lentinan on HLA-DR expression on thyrocytes was also examined in the culture preparations before and after 48 h of lentinan-stimulation. Although cultures prepared before the stimulation contained less than 0·5% HLA-DR positive cells, 10·3 ± 2·3% thyrocytes were positive for the HLA-DR antigen after 48 h of lentinan-stimulation.

Effect of anti-IL-1 antibody on TSA derived from thyrocytes. The anti-IL-1 antibody was tested on crude lentinan-stimulated thyrocyte culture supernatants. The results are shown in Table 2. The effect of the antibody was the same on the lentinan-stimulated thyrocyte culture supernatants as on the standard IL-1 derived from monocytes. In this experiment, an effect of normal rabbit serum on thymocyte proliferation was also included as a control. No significant effects were observed. The results suggest that the anti-IL-1 antibody reacts with determinants shared by thyrocyte-produced TSA and monocyte-produced IL-1.

Elimination of OKM1 positive cells. Considering the possibility of contaminating monocytes in thyrocytes cultures, cells were treated with OKM1 and rabbit complement. After this treatment, the culture preparations were negative for monocytes as determined by IF staining with OKM1 and non-specific esterase. No difference in the subsequent production of TSA on stimulation with lentinan was found (Table 3).

DISCUSSION

Recently, a role for thyrocytes in the development of autoimmune thyroid disease has been discussed. Several studies have suggested that thyrocytes are involved in the pathogenesis of the disease by interacting with T cells with the thyroid (Londei *et al.*, 1984; 1985; Davies, 1985). Our data presented here shows that IL-1 like activity was induced from normal human thyrocytes on stimulation with lentinan and could, therefore, strengthen this hypothesis.

Lentinan is a beta (1–3) glucan extract from the edible mushroom, lentinan eodes (Chihara *et al.*, 1970). This non-toxic polysaccharide has been found to augment immune responses to a variety of antigens and increase host resistance against murine and human tumour (DiLuzio *et al.*, 1979; Hamuro, Rollinghoff & Wagner, 1980). This agent has also been reported to stimulate murine and human macrophages to produce IL-1 (Hamuro *et al.*, 1980; Fruehauf, Bonnard & Herberman, 1982).

Thyrocytes did not spontaneously produce TSA. However, TSA was detected 24 h after stimulation with lentinan and reached its highest levels during the period from 24 to 48 h after stimulation. At that time, 10 ± 2·3% of thyrocytes were positive for HLA-DR antigen. Although the culture preparations contained less than 0·5% T cells, the antigen is likely to be induced on thyrocytes via production of gamma interferon by contaminating T cells (Todd *et al.*, 1985; Piccinini *et al.*, 1987). These phenomena could provide an important insight into a role for thyrocytes in the development of autoimmune thyroid disease because both secretion of IL-1 and expression of HLA-DR are activation signals for the T cells.

Before concluding that thyrocytes are capable of secreting TSA, careful consideration was given to exclude the possible contribution of monocytes, endothelial cells, fibroblasts and B cells to the results. In 5-day-old culture preparations, HLA-DR and $OKM1^+$ cells were contained less than 0·5%. Removal of $OKM1^+$ cells did not effect the subsequent production of TSA on stimulation with lentinan. It should be pointed out that any contaminating monocytes in the culture would be more than 1 week old, and monocytes in cultures for more than 48 h have been shown to have very low IL-1 producing capacity (Lepe-Zuniga & Crery, 1984). Culture preparations on day 5 contained less than 0·5% B cells and less than 1% fibroblasts and were negative for anti-factor VIII. Furthermore, to exclude the contribution of B cells and fibroblasts to the results, 5×10^3/ml B cells from peripheral blood from a healthy donor or 1×10^4/ml human fibroblasts prepared from samples of skin biopsy (5×10^3/ml and 1×10^4/ml corresponding to 0·5% and 1% of total cell population in thyroid cultures, respectively) were cultured and subsequent production of TSA on stimulation with 1·0 μg/ml lentinan was also studied; thymocytes incubated with TSA from these B cells or fibroblasts or with PHA alone incorporated 404 ± 22 ct/min, 323 ± 15 ct/min and 295 ± 26 ct/min, respectively. TSA produced in these cultures were much lower than that found in thyrocyte cultures.

It is not possible to totally exclude some contribution of other cell types to the IL-1 production by this type of experiment, but the data are consistent with the majority of the TSA derived from thyroid cultures being produced by thyrocytes.

Until recently, IL-1 was considered to be a strictly monocyte/macrophage product. Our findings indicate that an additional cell type, thyrocytes, also produce TSA. Whether TSA secreted from thyrocytes and IL-1 are biochemically identical will require further purification, physicochemical analysis, and detection of mRNA for IL-1 in thyrocytes to finally resolve this problem.

The role of TSA secreted by thyrocytes in the pathogenesis of autoimmune thyroid diseases remains speculative. The release of TSA by these cells may promote local immune responses in the thyroid, and thyrocytes may potentially serve as the principal activator of T cells that are infiltrating the thyroid as described by Davies (1985). The investigation of these problems may be expected to provide important insights into the pathogenesis of autoimmune thyroid disease.

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