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DIMETHYLSULFOXIDE MAINTAINS HUMAN THYROID CELLS IN SUSPENSION CULTURE, FACILITATING SYNTHESIS AND RELEASE OF THYROID HORMONE

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Usually, human thyrocytes in primary culture rapidly lose their thyroid function and fail to synthesize or release thyroid hormone after 3-5 days of culture. By culturing thyroid follicles obtained from patients with Graves' disease in medium supplemented with TSH and a low concentration of fetal calf serum (1%), thyrocytes can maintain thyroid function for several days.

We have found that the addition of dimethylsulfoxide to culture medium (1.7%) furthermore enhanced and maintained thyroid function (de novo synthesis and release of [^{125}I] thyroxine) for more than 13 days, probably by inhibiting dedifferentiation of thyrocytes. The present bioassay will be also useful for detecting thyroid stimulating immunoglobulin in patients with Graves' disease.

There are a number of in vitro bioassay methods for detecting TSH and thyroid-stimulating antibodies (TSAb) in Graves' disease (1). Most of these measure cyclic AMP generated in thyrocytes. Several methods measures 3,3'5'-triiodo-thyronine (T_3) released from cultured thyroid tissue or thyrocytes in a monolayer (2,3). However, there is no evidence that T_3 or thyroxine (T_4) released is synthesized de novo in thyrocytes in vitro. Needless to say, an ideal bioassay for TSAb would be one that measures end-products (T_3 or T_4) synthesized de novo and released into the culture medium.

Preliminary data obtained in our laboratory have revealed that human thyrocytes in primary culture rapidly lose their thyroid function and fail to synthesize or release thyroid hormone after 3-5 days of culture (data not shown). We therefore modified our culture medium by adding dimethylsulfoxide (DMSO) to prevent de-differentiation of functioning thyrocytes.

Materials and Methods

Suspension culture of thyroid follicular cells

Thyroid tissue (10-15 g) obtained by subtotal thyroidectomy from patients with Graves' disease was immediately transferred to ice-cold PRMI-1640 medium.

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These patients received 27 mg of iodide for 7-10 days before the operation. Informed consent was obtained from all subjects. The tissue was minced with scissors and washed with ice-cold Ca++- and Mg++- free Hanks' balanced salt solution (HBSS) and digested with 0.3 mg/ml collagenase (type II, Sigma Chemical Co., St. Louis, MO) and 5 mg/ml dispase (Godo-Shusei Co., Tokyo, Japan) in HBSS at 32°C for 30 min. The digested material was filtered through nylon mesh (100 mesh), and the undigested tissue fragments were processed in the same manner two more times. The pooled filtrate was centrifuged at 100 × g for 5 min, and the pellet was washed twice with HBSS. The pellet consisted mainly of follicles and a small amount of isolated cells.

The pellets were resuspended in RPMI-1640 medium (GIBCO, Grand Island, NY) supplemented with 1% fetal calf serum (FCS), insulin (8 μg/ml), hydrocortisone (10^{-8} M), transferrin (5 μg/ml), NaI (10^{-8} M), DMSO (1.7% vol/vol) and various concentrations of bovine thyrotropin (bTSH) (0-100 μU/ml, Sigma Chemical Co.), unless otherwise described. One milliliter of the suspension containing 2,000-3,000 follicles (about 10 μg of protein) was added to each well (2 cm^2) of 24-multiwell plates (Nunc, Roskilde, Denmark). These culture plates had been precoated with 0.5% agarose to prevent cell attachment (4). Cells were cultured in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. After 3 days of culture, 0.5 ml of fresh medium was added.

After 5 days of culture, 1 ml of medium was removed and the same volume of fresh medium containing about 2 × 10^5 cpm of $Na^{125}I$ was added, unless otherwise stated. After an additional 2 or 3 days of culture, medium and thyroid follicles were transferred to glass tubes and centrifuged at 1500 × g for 10 min. Conditioned medium (about 1.4 ml) was transferred to another glass tube, to which 100 μl of outdated human serum was added. Then, 200 μl of 50% trichloroacetic acid was added and centrifuged at 1500 × g for 10 min. The supernatant was aspirated and the pellet was resuspended with 2 ml of 5% TCA, and centrifuged again at 1500 × g for 10 min. This washing procedure was repeated once more. Then, ^{125}I radioactivity in the thyroid follicles and in the TCA precipitate (organic ^{125}I released into the medium) was counted in a γ-spectrometer. Since the protein content of thyrocytes in each well was almost equal after 7-8 days of culture, all data were expressed as cpm per well.

Thin-layer chromatography (TLC)

Thyroid follicles in quadruplicate culture were combined and homogenized in 0.3 ml of cold 0.11 M NaCl solution containing 30 mM Tris-HCl (pH 8.5). The homogenate (0.25 ml) was added to 0.25 ml of pronase solution (final concentration 0.17%, Seikagaku-Kogyo, Tokyo, Japan). After adding of one drop of toluene, digestion was performed at 37°C without shaking, according to the method of Inoue and Taurog (5). After 18 h of digestion, 0.1 ml of 4 N HCl solution was added and the reaction mixture was extracted with butanol (2 ml) twice as described previously (6). To the combined n-butanol extract, an equal volume of chloroform (~4 ml) was added and mixed well. The turbid mixture was extracted with 2 ml of 2 N NH_4OH solution twice. The combined extracts were lyophilized and organic ^{125}I was analyzed by TLC using a solvent system of n-butanol saturated with 2 N NH_4OH, as described previously (7). The recovery of radioactivity in the homogenate was approximately 50%.

In order to analyze organic ^{125}I released into the medium, the TCA-precipitate was dissolved in 0.5 ml of 0.1 N HCl solution. Then, the solution was extracted with n-butanol and the extracted organic ^{125}I was analyzed by TLC as described above. The recovery of radioactivity in the TCA-precipitate was approximately 50%.

Results

Effects of TSH and DMSO concentration on thyroid function

When human thyrocytes were cultured with TSH, DMSO enhanced TSH-stimulated thyroid function (incorporation of ^{125}I into thyroid follicles in suspension culture and release of organic ^{125}I into the medium) in a dose-dependent manner (Fig.1). The optimal DMSO concentration was 1-2% (vol/vol), whereas a higher

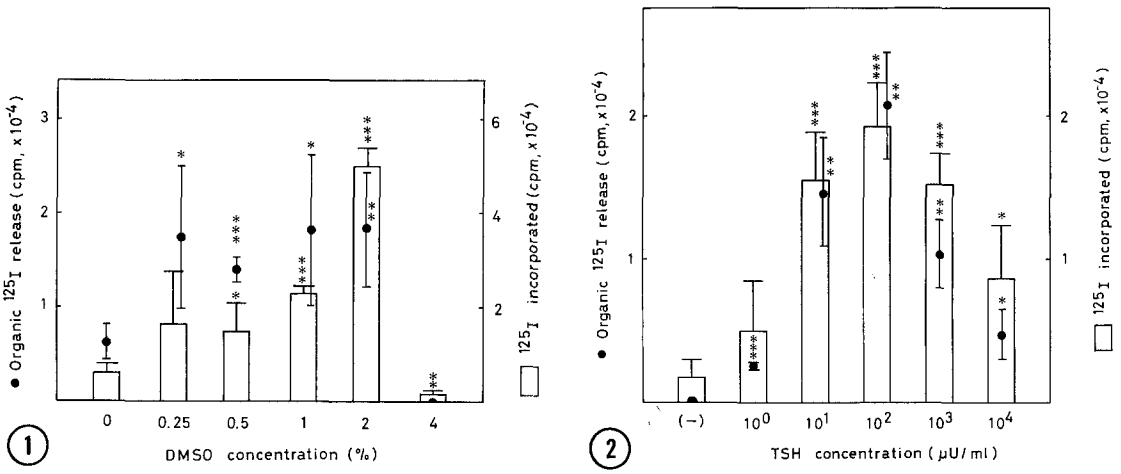


Fig. 1 Effect of DMSO concentration on thyroid function

Thyroid follicular cells were cultured in modified PRMI-1640 medium supplemented with 1% FCS, NaI (10^{-8} M), bTSH (100 μU/ml) and various concentrations of DMSO. After 5 days of culture, Na^{125}I (about 2 × 10^5 cpm) was added and after an additional 3 days of culture, ^{125}I incorporated into thyrocytes (clear columns) and organic ^{125}I released into the medium (solid cirles) were counted, as described in Materials and Methods. Data are means ± SD of quadruplicate cultures. * P<0.05, ** P<0.01, *** P<0.001, DMSO (−) vs. DMSO (+). Representative data from three experiments are shown.

Fig. 2 Effect of TSH concentration on thyroid function

Thyroid follicular cells were cultured in modified PRMI-1640 medium supplemented with 1% FCS, NaI (10^{-8} M), DMSO (1.7%) and various concentrations of TSH. After 5 days of culture, ^{125}I (2 × 10^5 cpm) was added to the culture medium and after an additional 3 days of culture, ^{125}I incorporated into thyrocytes (clear columns) and organic ^{125}I released into the medium (solid circles) were counted as described in Materials and Methods. Data are means ± SD of quadruplicate cultures. * P<0.05, ** P<0.01, *** P<0.001, TSH (−) vs. TSH (+). Representative data of three experiments are shown.

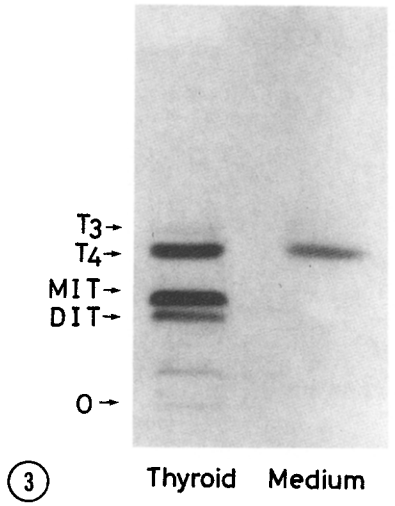
concentration (4%) was definitely inhibitory to thyroid function. Therefore, all cultures were performed in RPMI-1640 medium containing 1.7% DMSO.

TSH dose-dependently stimulated human thyroid follicles to incorporate ^{125}I and release organic ^{125}I into the medium. The minimum TSH concentration required to significantly stimulate organic ^{125}I release was 1 μU/ml (Fig.2). The maximal TSH effect occurred at 100 μU/ml, but supraphysiological concentrations of TSH (1 mU/ml-10 mU/ml) were rather inhibitory for thyroid function, probably reflecting desensitization to TSH.

Thin layer chromatography studies revealed that ^{125}I within the thyroid follicular cells was composed of monoiodotyrosine (MIT), diiodotyrosine (DIT), T_3 and T_4, whereas organic ^{125}I released into the medium was composed mainly of T_4 but not of iodotyrosines (Fig.3).

Effect of DMSO on time course of thyroid function

When human thyroid follicles were cultured in medium containing 1% FCS and 100 μU/ml bTSH with or without DMSO for 1,3,5,7,9 and 11 days and then thyroid function was studied, thyrocytes cultured in the absence of DMSO gradually lost



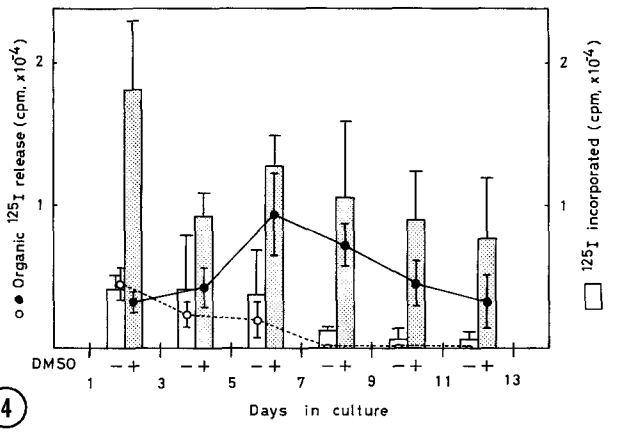


Fig. 3 Analysis of ^{125}I compounds in the thyrocytes and medium by TLC

Thyroid follicular cells were cultured in modified RMPI-1640 medium supplemented with 1% FCS, NaI (10^{-7} M), bTSH (100 μU/ml) and DMSO (1.7%). After 5 days of culture, ^{125}I (1×10^6 cpm) was added to the culture medium and after an additional 2 days of culture, ^{125}I incorporated into thyrocytes and organic ^{125}I released into the medium were counted. Then, ^{125}I compounds were extracted and analyzed by TLC using a solvent system of n-butanol saturated with 2 N NH_4OH. MIT: monoiodotyrosine, DIT: diiodotyrosine.

Fig. 4 Effect of DMSO on time course of thyroid function

Thyroid follicular cells were cultured in modified RPMI-1640 medium supplemented with 1% FCS, NaI (10^{-8} M) and bTSH (100 μU/ml) in the presence (▨, ●—●) or absence (□, ○—○) of DMSO (1.7%) for 1,3,5,7,9 and 11 days. Thereafter, ^{125}I (about 2×10^5 cpm) was added to each well and after an additional 2 days of culture, ^{125}I incorporated into thyrocytes (columns) and organic ^{125}I released into the medium (circles) were counted as described in Materials and Methods. Data are means ± SD of quadruplicate cultures. Representative data of three experiments are shown.

their ability to incorporate ^{125}I and release organic ^{125}I into the medium (Fig.4). Even though these cells were cultured with TSH, they completely lost their thyroid function after 7 days of culture, whereas thyrocytes cultured in the presence of DMSO and TSH were capable of incorporating ^{125}I and releasing organic ^{125}I into the medium throughout the culture period for up to 13 days.

Since thyrocytes cultured without TSH incorporated little radioactivity and released only a trace amount of organic ^{125}I after 5 days of culture (data not shown), $Na^{125}I$ was usually added after 5 days of culture.

Effect of FCS concentration on thyroid function

Fig.5 shows the effects of FCS on thyroid function in thyroid follicles cultured with 100 μU/ml bTSH in the absence or presence of DMSO. The addition of FCS produced a deterioration of thyroid function in a concentration-dependent manner (1-10%): de novo synthesis and release of organic ^{125}I was not observed when thyrocytes were cultured in medium containing 5-10% FCS. This dose-dependent inhibitory effect of FCS on thyroid function was greatly prevented by the addition of DMSO (1.7%) into the medium (Fig.5).

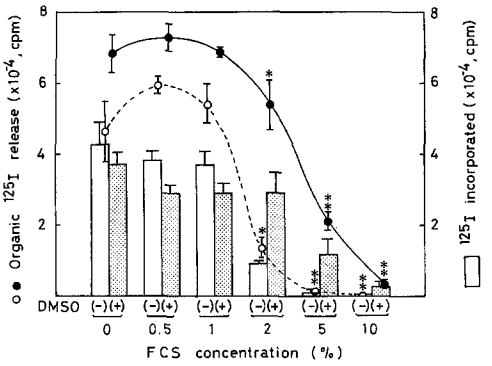


Fig. 5 Effect of FCS concentration on thyroid function
Thyroid follicular cells were cultured in modified RPMI-1640 medium with or without DMSO (1.7%) and various concentrations of FCS. After 5 days of culture, ^{125}I (2 × 10^5 cpm) was added to the culture medium and after an additional 3 days of culture, ^{125}I incorporated into thyrocytes (columns) and organic ^{125}I released into the medium (○ , ●) were counted as described in Materials and Methods. Data are means ± SD of quadruplicate cultures. Representative data of four experiments are shown. * P<0.01, ** P<0.001, FCS(-) vs. FCS (+).

Discussion

DMSO has a number of pharmacological and biological effects (8). Its cryoprotective action (10% vol/vol) is well known to cell biologists for the storage of cultured cells in liquid nitrogen. Furthermore, DMSO (2% vol/vol) enhances the differentiation of leukemic cells to mature myelocytes (9). In normal cells, DMSO (1.5%) prevents dedifferentiation; for example, cultured adult rat hepatocytes can be maintained and made to secrete albumin for more than 40 days (10). This was also the case for human thyroid follicular cells in suspension culture. The addition of DMSO to the culture medium preserved thyroid function for up to 13 days, whereas thyroid function diminished gradually in thyrocytes cultured without DMSO, even though the medium contained a low concentration of FCS together with TSH.

For some unknown reason, high concentrations of FCS in the culture medium deteriorate thyroid function. When human thyrocytes were cultured in monolayer in medium supplemented with 10% FCS, thyrocytes were able to release T_3 in response to TSH (11), but rapidly lost their ability to concentrate and organify iodide, suggesting that T_3 secretion did not rely on de novo T_3 synthesis but rather corresponded to the release of preformed T_3 from intracellular stores (11). These findings are consistent with ours in suspension culture (Fig.5). To prevent de-differentiation of human thyrocytes, they should be cultured in medium supplemented with low concentrations of FCS or even without FCS, as reported for porcine and sheep thyrocytes (12,13).

By culturing human thyroid follicles in medium containing a low concentration of FCS (~1.0%) and DMSO (1.7%), we have established a very sensitive bioassay for TSH, in which cultured thyrocytes incorporate ^{125}I, synthesize de

novo iodotyrosines and iodothyronines and secrete mainly [^{125}I]T_4 and [^{125}I]T_3 into the medium. This was also the case with IgG obtained from sera of patients with Graves' disease (data not shown). Since our present bioassay detects end-products of TSH and TSAb, it would be an ideal bioassay for TSAb. In addition, our bioassay is very simple and cost-effective. After addition of inorganic ^{125}I into the medium, organified ^{125}I released into the medium is just counted. Our preliminary data indicate that the present bioassay is also very sensitive to TSAb. Therefore, the bioassay, which measures end-products of the thyroid stimulators, will be useful for detecting TSAb in the serum of patients with Graves' disease.

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