Effects of 3,5,3′-triiodothyronine on collagen synthesis by cultured human skin fibroblasts

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Addition of 3,5,3′-triiodothyrinine (T3) ($10^{-9} - 10^{-7}$ M) to the media of cultured skin fibroblasts was shown to decrease the amount of newly synthesized soluble proteins, including collagen. Polyacrylamide gel electrophoresis of newly synthesized collagen demonstrated that addition of the hormone did not affect the processing of the molecule.

Collagen *Fibroblast* *Thyroid hormone* *Triiodothyronine*

1. INTRODUCTION

Collagen, the most abundant structural protein of connective tissue, represents well over 70% of the dry weight of human skin [1]. The effects of various factors on its biosynthesis have been reviewed [2]. Thyroid hormones are known to modulate the cellular metabolism of most of the mammalian tissues and the existence of nuclear receptors for 3,5,3′-triiodothyronine (T3) has been demonstrated in different types of tissues including human fibroblasts [3].

A possible role for thyroid hormones in the control of fibroblast growth has been suggested [4]. Moreover, alterations in skin and connective tissues are known to occur in thyroid dysfunctions [5]. However, despite these data and the accessibility of human fibroblasts to investigators, the effects of thyroid hormones on the biosynthesis of collagen by these cells have not yet been systematically studied. This prompted us to investigate an 'in vitro' system as a possible model for the study of thyroid hormone action at a cellular level.

2. MATERIALS AND METHODS

2.1. *Chemicals*

Highly purified collagenase (form III) was purchased from Advance Biofacture; β-aminoproprionitrile (B-APN), *N*-ethylmaleimide (NEM), EDTA, dithiothreitol (DTT) were from Sigma. Phenylmethanesulfonyl fluoride (PMSF) was from Fluka. Pepsin was from Worthington. SDS was purchased from Biorad.

High purity grade T3 was obtained from Henning Berlin; L-[5-^{3}H]proline (1 mCi/ml) was from New England Nuclear.

2.2. *Culture medium*

Dulbecco's modified Eagle's medium (DMEM) was obtained from Gibco Europe. Fetal calf serum (FCS) was purchased from Continental Pharma.

Thyroidectomized calf serum (TCS): a newborn calf was thyroidectomized at age 10 days and received, 10 days later, a dose of 100 mCi ^{131}I. Serum total and free T3 and T4 were monitored weekly. Eleven weeks after thyroidectomy free T3 and free T4 dropped below 0.4 and 0.6 pg/ml, respectively, while total T3 was 13 ng/dl and total T4 0.08 μg/dl. The animal was then killed and the blood collected. The serum, aliquoted and frozen, is subsequently referred as TCS.

2.3. *Cell culture*

Primary culture of human skin fibroblasts were initiated from skin punch biopsies performed on healthy young adults. Fibroblasts were grown in

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DMEM supplemented with penicillin (100 units/ml), streptomycin (100 units/ml), 10% FCS, in an incubator at 37°C in humidified 95% air/5% CO_2. Subculture was performed using 0.25% trypsin and 0.05% EDTA. All fibroblasts were studied between the 5th and 10th passage.

At the start of the experiments, cells were plated in 25-cm^2 Petri dishes (Nunc) and maintained in DMEM containing 10% FCS until confluency. The cells were then preincubated for 48 h with 10% TCS–DMEM medium with or without various amounts of T3. These media were then replaced by similar media supplemented with ascorbate (50 μg/ml), B-APN (50 μg/ml) and 20 μCi/ml of L-[5-^{3}H]proline. Two dishes were used for each experimental condition.

2.4. *Collagen measurement*

After 24 h labelling, the media were decanted from the cell layer, and protease inhibitors (NEM and PMSF) were added to final concentrations of 250 and 50 μM, respectively. Fibroblasts were trypsinized and counted.

Aliquots of the media were taken for determination of total radioactivity. The culture media were then dialysed extensively against 0.05 M Tris, 0.15 M NaCl buffer, at 4°C.

After dialysis, aliquots of the dialysates were counted in an LKB liquid scintillation counter. The non-dialysable associated radioactivity was used as an indication of the total amount of protein synthesized by fibroblasts during the 24 h period of labelling.

Similarly, the amount of newly synthesized collagen was determined by measurement of the radioactivity assoeiated with the 'non-dialysable, collagenase-digestible' material as in [6].

2.5. *Thyroid hormone assays*

Commercial RIA kits were used for the measurements of total T3, total T4 (Techland®, Liège), free T3 and free T4 (Lepetit® , Milan).

2.6. *Polyacrylamide gel electrophoresis*

Dialysed media were precipitated with ammonium sulfate at a final concentration of 40% saturation. The pellet was dissolved in a 20% ammonium sulfate buffer and respun. The precipitate was redissolved and dialysed against the same buffer. After addition of carrier collagen, the samples were submitted to enzymatic digestion with pepsin and collagenase and the resulting aliquots lyophilised. The dessicated material was dissolved in the electrophoresis buffer, with or without DTT, heated for 10 min at 60°C in a water bath and loaded on a 6.25%, polyacrylamide slab gel containing 0.5 M urea and 0.1% SDS. The gels were stained with Coomassie blue and treated for fluorography [7]. The dried gels were exposed to X-ray films for 2 weeks at −70°C.

3. RESULTS

Experiments were performed on fibroblasts from 4 healthy adults. The effect of increasing T3 concentrations (3×10^{-9}, 7.5×10^{-9}, 7.5×10^{-8} M) in the culture medium was studied. Under these conditions, measured fT3 concentrations were 24.5×10^{-12}, 82.5×10^{-12}, 148.4×10^{-12} M, respectively.

3.1. *Effect of TCS on protein and collagen synthesis*

Eight different experiments were performed to compare protein synthesis in TCS *vs* FCS conditions. Inhibition of total protein synthesis with FCS (range: 8–46% of control with TCS) and collagen synthesis (range: 10–29% of control with TCS) was systematically observed.

3.2. *Effect of T3 on cell growth*

Addition of T3 to the culture media for 3 days after confluence did not affect cell viability since

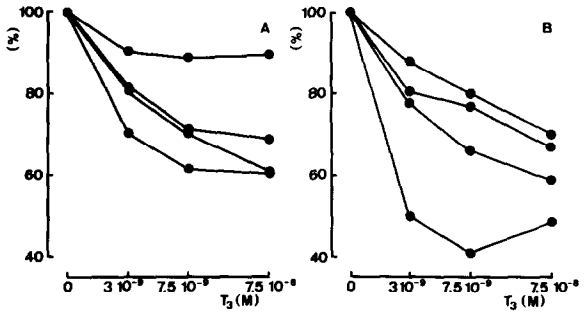

Fig.1. Synthesis of total protein (A) and collagen (B) expressed as percentage of control. Three doses (3×10^{-9}, 7.5×10^{-9}, 7.5×10^{-8} M) of thyroxine were added to culture media for two days after cell confluency. [^{3}H]Proline was added for 24 h and synthesis measurements were performed as described in section 2.

the number of living cells per dish was not different in the presence or the absence of T3 (10^{-9} M–10^{-7} M). This number was approx. 10^6 cells per dish. Moreover, no change occurred in fibroblast morphology when cells were fed with culture medium supplemented with T3.

3.3. *Effect of T3 on protein synthesis*

When cells were incubated in T3 supplemented medium, inhibition of total protein synthesis was consistently observed (fig.1). However, while in any given subject the magnitude of the inhibiting effect usually appeared higher with the larger doses of T3, no clearcut dose-response relationship could be defined.

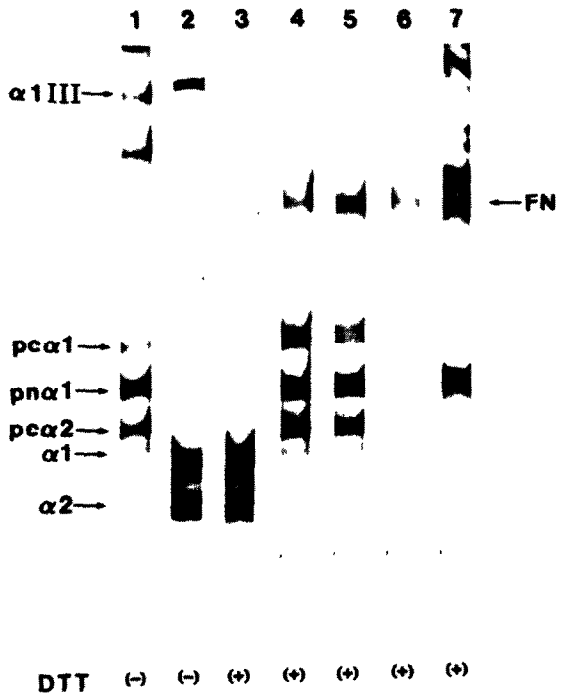

Fig.2. Fluorescent autoradiogram of [^{3}H]proline labelled proteins from skin fibroblast media. SDS–PAGE was performed on 6.5% slab gels in the absence (lane 1,2) and the presence of DTT (lane 3–6). Gel patterns correspond to that given by control media with the exception of lane 5 (medium + 7.5×10^{-8} M T3). Undigested media were applied to lanes 1,4,5; pepsin-resistant material is shown in lane 2,3; fibronectin remaining after collagenase digestion is shown in lane 6; lane 7 exhibits the pattern of radiolabelled markers (FN and pn 1, in the presence of DTT.

3.4. *Effect of T3 on collagen synthesis*

A reduction in the amount of newly synthesized collagen was observed in all cases in the presence of T3 (fig.1.). The inhibiting effect of T3 on collagen synthesis (16–53%) was more marked than on total protein synthesis, with an indication of a dose-response relationship.

Qualitative changes were studied by SDS-polyacrylamide gel electrophoresis (SDS–PAGE). Typical fluorographic profiles are shown in fig.2. The main products secreted by fibroblasts into the media were pn 1 (I), pc 1 (I) and pc 2 (I) collagens (lane 1). In our experiments some type III collagen was also secreted: a protein fraction from pepsin digested media, identified as 1 (III) was revealed at the top of the gel (lane 2) but was no longer evident in a DTT-treated sample (lane 3). Fibronectin was also secreted by fibroblasts since a collagenase-resistant material was visualized after DTT reduction (lane 6).

In the 4 cases studied using PAGE, the electrophoretic and autoradiographic patterns were similar for media from fibroblasts grown in the absence (lane 4) or presence (lane 5) of T3.

4. DISCUSSION

It has been suggested that thyroid hormones are involved as regulating factors in the metabolism of proteins and collagen [8–12] but few studies have previously been published describing thyroid hormone effects on their synthesis.

Depending on the preexisting thyroid status, thyroid hormones have been reported to increase [11] or decrease [12] the synthesis of collagen, to have no effect on or to increase the catabolism of collagen as well as to accelerate the conversion of soluble to insoluble collagen [11]. Variations in the urinary excretion of hydroxyproline have been reported in hypo- and hyperthyroid patients [14] as well as in rats made experimentally hypo- or hyperthyroid [12,13]. These observations strongly suggest that collagen metabolism is indeed affected by thyroid hormones in vivo.

Human fibroblasts are known to possess nuclear receptors for T3 [3] and to actively metabolize thyroid hormones [16]. Some studies exploring low density lipoprotein degradation [17] and glycosaminoglycan accumulation [18] were performed using human fibroblasts in vitro.

However, no demonstration has yet been made of the direct effect of T3 or T4 on the biosynthesis of collagen by cultured skin fibroblasts.

Our data consistently demonstrate a decrease in the total amount of protein synthesis and secretion by human fibroblasts in the presence of non-pharmacological concentrations of T3 in the culture medium. The synthesis of collagen, the most important protein synthesized by fibroblasts in culture, was specifically depressed by T3 in normal skin fibroblasts in vitro. Moreover, with fibroblasts for 3 out of the 4 subjects studied, a dose-response effect could be demonstrated. Analytical PAGE and fluorography failed to demonstrate any qualitative change of the protein synthesized by fibroblasts grown with or without T3. The model we propose is particularly convenient as it fulfills 3 important criteria for an in vitro test for the study of thyroid hormone effects.

First, fibroblasts are easily obtained from skin biopsies. Second, the recovery of the radioactive collagen secreted into the medium and the enzymatic tests for its quantification do not require sophisticated techniques. Last, in this experimental system, the independent variable is well controlled. Incubation of cells in medium treated to eliminate thyroid hormones is known to enhance the sensitivity of different in vitro systems used for the study of thyroid hormone action [18–20]. We preferred the use of medium in which serum from radically thyroidectomized calf had been introduced and supplemented with T3 only. The techniques using activated charcoal or resins to produce T3 and T4 free serum also unselectively exclude other factors potentially important in the metabolism under study.

Moreover, our procedure insures that the biological effects are indeed observed in the presence of the metabolically relevant fraction of T3 (free T3) as it is precisely determined.

Thus, the above-mentioned advantages of our model permit the study of the direct influence of T3 on the biosynthesis of collagen by human skin fibroblasts. This new tool is now available for both experimental endocrine research and for clinical investigation, such as the study of thyroid hormone resistance syndromes.

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REFERENCES

[1] Bauer, E.A. and Uitto, J. (1979) Int. J. Dermatol. 18, 251–270.
[2] Müller, P.K., Kirsch, E., Gauss-Müller, V. and Krieg, T. (1981) Mol. Cell. Biochem. 34, 73–85.
[3] DeGroot, L.J., Refetoff, S., Bernal, J., Rue, P.A. and Coleoni, A.H. J. Endocrinol. Invest. 1, 79–88. 1, 79–88.
[4] Vogelaar, J.P.M. and Erlichman, E. (1936) Am. J. Cancer 26, 358–367.
[5] Freedberg, I. and Kohn, L. in: The Thyroid (Werner, S. and Ingbar, S. eds) pp.636–654 (851–855), Harper and Row.
[6] Peterkofsky, B. and Diegelmann, R. (1971) Biochemistry 10, 988–994.
[7] Bonner, W.M. and Laskey, R.A. (1974) Eur. J. Biochem. 46, 83–88.
[8] Thieblot, L., Berthelay, J., Blaise, S. and De Laguillaumie, B. (1963) C.R. Soc. Biol. 157, 543–545.
[9] Langner, R.O. and Fuller, G.C. (1973) Atherosclerosis 17, 463–469.
[10] Mehregan, A.H. and Zamick, P. (1974) J. Cutaneous Pathol. 1, 113–116.
[11] Kuberasampath, T. and Bose, S.M. (1979) Agents Actions 9, 503–509.
[12] Kivirikko, K.I., Laitinen, O., Aer, J. and Halme, J. (1967) Endocrinology 80, 1051–1061.
[13] Drozdzm, M., Kucharz, E., Grucka-Mamczar, E. (1979) Endokrinologie 73, 105–111.
[14] Mosekilde, L., Christensen, M.S., Melsen, F. and Rensen, S. (1978) Acta Endocrinol. 87, 743–750.
[15] Kivirikko, K.I., Laitinen, O. and Lamberg, B.A. (1965) J. Clin. Endocrinol. 25, 1347–1352.
[16] Refetoff, S., Matalon, R. and Bigazzi, M. (1972) Endocrinology 91, 934–947.
[17] Chait, A., Bierman, E.L. and Albers, J.J. (1979) J. Clin. Endocrinol. Metab. 48, 887–889.
[18] Smith, T.J., Horwitz, A.L. and Refetoff, S. (1981) Endocrinology 108, 2397–2399.
[19] Smith, T.J., Murata, Y., Horwitz, L., Philipson, L. and Refetoff, S. (1982) J. Clin. Invest. 70, 1066–1073.
[20] Chait, A., Kanter, R., Green, W. and Kenny, M. (1982) J. Clin. Endocrinol. Metab. 54, 767–772.
[21] Samuels, H.H. (1978) in: Receptors and Hormone Action, vol.III, Academic Press, New York.