

# Triiodothyronine stimulates proliferation of osteoblast-like cells in serum-free culture

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Primary rat osteoblast-like cells (Ob) were grown under strictly serum-free conditions for up to 20 days. In the presence of triiodothyronine ( $T_3$ ) at concentrations of 0.01 and 0.1 nM, Ob proliferation was enhanced. Moreover, a decrease of alkaline phosphatase (AP) activity, a differentiation marker for Ob, was prevented, whereas protein synthesis (collagen and noncollagen protein) was decreased.  $T_3$  at much higher concentration (10 nM) had no significant effect on cell proliferation and matrix formation but decreased AP activity disproportionately. Thus,  $T_3$  at close to physiological concentrations stimulates growth and maintains differentiation of Ob.

Serum-free culture; Collagen synthesis; Alkaline phosphatase; Triiodothyronine; (Osteoblast-like cell)

## 1. INTRODUCTION

Thyroid hormones are major regulators of skeletal growth and maturation [1]. They are required for normal growth in vivo and also contribute to the replication of cells in vitro [2]. Changes in thyroid function are associated with alterations in bone metabolism [3]. In hypothyroidism, bone turnover is slow and bone growth and maturation are disturbed. On the other hand, hyperthyroidism is associated with an increase in bone formation often accompanied by a negative calcium balance [3,4]. On the one hand, thyroid hormones are required for release of growth hormone and subsequently the synthesis of insulin-like growth factors (IGFs) in the liver [5], the major anabolic hormone for bone and cartilage [6]. On the other, thyroid hormones have a direct stimulatory effect on cartilage growth and maturation [7].  $T_3$  has not yet been shown to influence proliferation of osteoblasts, but rather to stimulate

osteoclastic bone resorption [8]. Using a sensitive cell culture system for Ob, we report here that  $T_3$  directly stimulates growth and affects other Ob parameters.

## 2. MATERIALS AND METHODS

Ob from calvaria of newborn rats (ZUR:SIV, formerly Sprague Dawley) were subjected to sequential digestion with bacterial collagenase and inoculated into serum-free medium as in [9].  $T_3$  (3,3',5-triiodo-L-thyronine sodium salt, Sigma) was added at the beginning of the culture period lasting 19–21 days. Cell growth was monitored 'on line' as described [9].

AP activity was determined according to [10] and protein content by the method of Bradford [11] using BSA as a standard.

For determination of collagen synthesis, culture dishes (35 mm) were pulsed with 3  $\mu$ Ci/ml [ $^3$ H]proline (20 Ci/mmol, Amersham) in the presence of 50  $\mu$ g/ml ascorbic acid. After labeling, cells were scraped off and pooled with their respective medium and stored at  $-20^\circ\text{C}$ . Following sonification of the cell suspension, trichloroacetic

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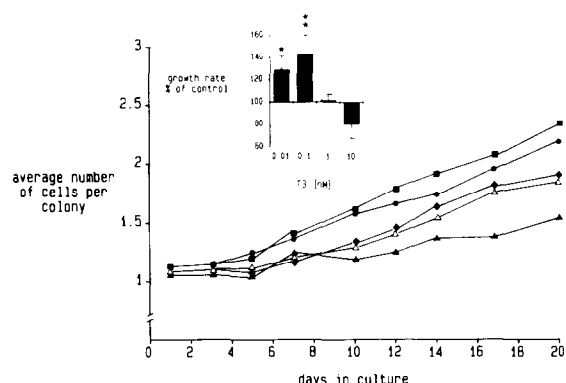


Fig.1. Effects of  $T_3$  on proliferation of rat Ob. Single cells were inoculated at about  $1.3 \times 10^5$  cells/ml per culture dish (35 mm).  $T_3$  was present from the beginning of the culture period which lasted for 19–21 days ( $37^\circ\text{C}$ , 90%  $\text{N}_2$ , 5%  $\text{CO}_2$ , 5%  $\text{O}_2$ ). Cell growth was monitored on line by counting the percentage of colonies containing a distinct number of cells on the same dish three times a week. The average number of cells per colony was plotted as a function of time. The final  $T_3$  concentrations in the medium were: ( $\Delta$ ) 0 nM (control), ( $\bullet$ ) 0.01 nM, ( $\blacksquare$ ) 0.1 nM, ( $\blacklozenge$ ) 1.0 nM, ( $\blacktriangle$ ) 10.0 nM. The dots represent the means of four culture dishes from the same Ob preparation. (Inset) The growth curves were described by linear regression lines with correlation coefficients  $r \geq 0.95$ . The resulting slopes of these lines were compared to that obtained in the absence of  $T_3$  (100%). Each point represents the mean  $\pm$  SD of three proliferation assays as outlined above derived from independent cell preparations. Significantly different from control: \* at  $p < 0.05$ , \*\* at  $p < 0.01$ .

acid was added to give a final concentration of 10% (w/v). After centrifugation and repeated precipitation, the protein solution was assayed for [ $^3\text{H}$ ]proline incorporation into collagen (CP) and noncollagen protein (NCP) using bacterial collagenase (Sigma, type VII) according to [12]. Instead of acid hydrolysis of the acid-precipitated NCP, soluene-350 (Packard) was used.

### 3. RESULTS

Rat Ob proliferate selectively into cell clusters of clonal origin under strictly serum-free culture conditions, in viscous medium, containing 0.8% methylcellulose and 0.25% delipidated bovine serum albumin [13].

$T_3$  (0.1 and 0.01 nM) stimulated Ob proliferation, an effect that became evident only after 5–6 days of culture (fig.1). In contrast, 10 nM  $T_3$  was inhibitory when compared to hormone-free control cultures. In the absence of  $T_3$ , the basal growth rate of control cultures in all experiments was small but significant. The same tendency was seen when the total protein content per culture dish was determined (not shown).

To determine whether  $T_3$  has any effect on Ob specific parameters, AP activity and collagen synthesis were measured: whereas AP activity decreased in cultures without  $T_3$ , the hormone elicited dose-dependent biphasic effects (table 1). AP activity in control and  $T_3$  cultures

Table 1  
Alkaline phosphatase activity in response to  $T_3$  in cultured rat Ob cells

[ $T_3$ ] (nM)	AP activity ( $\mu\text{mol}/\text{mg}$ protein per h)			
	5 days	12 days	17 days	20 days
0	$8.3 \pm 0.9$	$13.4 \pm 1.6^c$	$9.9 \pm 1.6$	$10.0 \pm 0.2$
0.01	$7.6 \pm 0.5$	$10.9 \pm 1.0$	$13.9 \pm 1.2^{ac}$	$12.1 \pm 0.8^c$
0.1	ND	$12.1 \pm 1.3$	$12.8 \pm 0.8^a$	$11.5 \pm 0.4^a$
1.0	ND	$11.6 \pm 0.9$	$8.7 \pm 0.8$	$10.0 \pm 0.8$
10.0	$8.7 \pm 0.3$	$8.7 \pm 0.5^b$	$3.6 \pm 0.4^{bd}$	$4.4 \pm 0.6^{bd}$

Cells were cultured for the indicated times and alkaline phosphatase (AP) activity was measured by the cleavage of *p*-nitrophenylphosphate to *p*-nitrophenol at  $37^\circ\text{C}$  and pH 10.2. Two experiments ( $n = 6$ , mean  $\pm$  SD; ND, not determined). Significantly different from controls at the corresponding times: <sup>a</sup>  $p < 0.05$ , <sup>b</sup>  $p < 0.01$ ; significantly different from values in cultures of corresponding  $T_3$  concentration at day 5: <sup>c</sup>  $p < 0.05$ , <sup>d</sup>  $p < 0.01$ .

Table 2

Effect of  $T_3$  on the incorporation of [ $^3H$ ]proline into collagen protein (CP) and non-collagen protein (NCP) in cultured rat Ob

[ $T_3$ ] (nM)	dpm/mg protein		Relative collagen synthesis (%)
	CP	NCP	
0	8033 $\pm$ 576	35700 $\pm$ 2115	4.0 $\pm$ 0.3
0.01	3534 $\pm$ 201 <sup>a</sup>	21599 $\pm$ 1907 <sup>a</sup>	2.9 $\pm$ 0.3 <sup>a</sup>
0.1	4900 $\pm$ 217 <sup>a</sup>	25526 $\pm$ 2305 <sup>a</sup>	3.4 $\pm$ 0.2
1.0	8113 $\pm$ 406	30345 $\pm$ 2812	4.7 $\pm$ 0.3
10.0	7872 $\pm$ 284	30167 $\pm$ 3007	4.6 $\pm$ 0.3

Cell cultures were pulsed at day 14 for 48 h and assayed as described in section 2. Values were corrected for the relative abundance of proline in CP and NCP as follows [21]: (dpm CP  $\times$  100)/([5.4  $\times$  dpm NCP] + dpm CP). 2 experiments ( $n = 6$ , mean  $\pm$  SD); <sup>a</sup> significantly different from control,  $p < 0.05$

(0.01–1.0 nM) increased from day 5 to 12. In the absence of  $T_3$  the highest AP activity was observed at day 12 with a decrease thereafter, whereas enzyme activity was maintained or even further increased by low concentrations of  $T_3$ . In contrast, 10 nM  $T_3$  decreased enzyme activity steadily below control values; 1 nM  $T_3$  had no effect, on both cell proliferation (fig.1) and AP activity.

Effects of  $T_3$  on collagen synthesis were investigated by pulsing with [ $^3H$ ]proline. Incorporation into CP and NCP was significantly decreased to 70 and 60%, respectively, of control in the presence of 0.01 and 0.1 nM  $T_3$  (table 2). Moreover, the relative amount of collagen protein synthesis decreased to 78% of controls in the presence of 0.01 nM  $T_3$ . Concentrations higher than 0.1 nM did not affect these parameters significantly.

#### 4. DISCUSSION

Ob were previously shown to sediment to the bottom of the dish and to remain there selectively as cells of spherical morphology in serum-free, viscous medium. Since they proliferate into clusters of clonal origin, colony growth can be monitored throughout the entire culture period on the very same dish. This culture system yields a

very sensitive and specific proliferation assay for testing the mitogenic potential of hormones affecting bone [9].

Under our culture conditions, proliferation of primary Ob is stimulated by  $T_3$  at close to physiological concentrations (0.1 and 0.01 nM). These concentrations in vitro may well reflect the free  $T_3$  concentration in serum, where most is bound to thyroxine-binding globulin. In our experiments, higher concentrations of  $T_3$  had no stimulatory, but rather inhibitory effects on cell growth. Stimulation became evident only 5–6 days after inoculation. Compared to insulin and IGF I under the same experimental conditions this corresponds to a delay of 2–3 days [9].

[ $^3H$ ]Proline incorporation into CP and NCP was decreased inversely to the stimulation of cell proliferation by  $T_3$  when compared to controls, although the relative amount of collagen synthesis was significantly decreased only by 0.01 nM  $T_3$ . It has been shown for several cell types that synthesis of CP and NCP is decreased during growth [14]. In organ cultures of rat calvaria it is apparently difficult to ascertain an effect on matrix formation by  $T_3$  [15].

Concentrations of 10 nM  $T_3$  had no significant effect on Ob proliferation and matrix formation, but decreased AP activity. High activity of the latter enzyme is considered to represent a marker of Ob differentiation [16]. It is, therefore, of interest that low concentrations of  $T_3$  prevented a decrease of enzyme activity in long-term Ob cultures, as has been reported in the osteoblast-like osteosarcoma cells, ROS 17/2.8 [17]. Whether this represents a higher average AP activity per cell or increased activity in a younger subpopulation of Ob, due to increased proliferation, cannot be decided.

The present results show an increased proliferation as well as differentiation of primary rat Ob in vitro by  $T_3$  at physiological concentrations. This is in keeping with nuclear thyroid hormone receptors, which have been demonstrated in ROS 17/2.8 cells [18]. Moreover, physiological concentrations of  $T_3$  were shown to increase ornithine decarboxylase activity in Ob cultures [19]. Cellular growth and differentiation have often been associated with the activation of ornithine decarboxylase.  $T_3$  also increases bone Gla-protein in ROS 17/2.8 cells [18] which is considered as an indicator of bone turnover, i.e. of bone formation [20].

We conclude that  $T_3$  affects bone in three ways. (i)  $T_3$  is an important regulator of growth hormone synthesis and secretion which in turn determines IGF I levels in serum [2]. (ii)  $T_3$  at physiological concentrations enhances Ob proliferation as well as the response of Ob to parathyroid hormone [19]. (iii)  $T_3$  at higher concentrations stimulates the release of  $^{45}\text{Ca}$  from bone in organ culture [8]. Physiological concentrations of  $T_3$ , therefore, stimulate bone formation and higher concentrations bone resorption.

## REFERENCES

- [1] Raisz, L.G. and Kream, B.E. (1981) *Annu. Rev. Physiol.* 43, 225–238.
- [2] Bouillon, R. (1986) *Q. J. Med.* 232, 737–752.
- [3] Mundy, G.R. and Raisz, L.G. (1979) *Miner. Electrolyte Metab.* 2, 285–292.
- [4] Krane, S.M., Brownell, G.L., Stanbury, J.B. and Corrigan, H. (1956) *J. Clin. Invest.* 35, 874–887.
- [5] Schalch, D.S., Heinrich, U.E., Draznin, B., Johnson, C.J. and Miller, L.L. (1979) *Endocrinology* 104, 1143–1151.
- [6] Schoenle, E., Zapf, J. and Froesch, E.R. (1982) *Nature* 296, 252–253.
- [7] Burch, W.M. and Lebovitz, H.E. (1982) *Endocrinology* 111, 462–468.
- [8] Mundy, G.R., Shapiro, J.L., Bandelin, J.G., Canalis, E. and Raisz, L.G. (1976) *J. Clin. Invest.* 58, 529–534.
- [9] Ernst, M. and Froesch, E.R. (1987) *Calcif. Tissue Int.* 40, 27–34.
- [10] Schmid, C., Steiner, T. and Froesch, E.R. (1983) *Calcif. Tissue Int.* 35, 578–585.
- [11] Bradford, M.M. (1976) *Anal. Biochem.* 72, 248–254.
- [12] Peterkofsky, B. and Diegelmann, R.F. (1971) *Biochemistry* 10, 988–994.
- [13] Chen, R.F. (1967) *J. Biol. Chem.* 242, 173–181.
- [14] Peterkofsky, B. (1972) *Arch. Biochem. Biophys.* 152, 318–328.
- [15] Canalis, E. (1983) *Endocr. Rev.* 4, 62–77.
- [16] Rodan, G.A. and Rodan, S.B. (1983) in: *Bone and Mineral Research/Annual 2* (Peck, W.A. ed.) vol.2, pp.244–285, Elsevier, Amsterdam, New York.
- [17] Sato, K., Han, D.C., Fujii, Y., Tsushima, T. and Shizume, K. (1987) *Endocrinology* 120, 1873–1881.
- [18] Rizzoli, R., Poser, J. and Burgi, U. (1986) *Metabolism* 35, 71–74.
- [19] Schmid, C., Steiner, T. and Froesch, E.R. (1986) *Acta Endocrinol.* 111, 213–216.
- [20] Hauschka, P.V., Lian, J.B. and Gallop, P.M. (1975) *Proc. Natl. Acad. Sci. USA* 72, 3925–3929.
- [21] Diegelmann, R.F. and Peterkovsky, B. (1972) *Dev. Biol.* 28, 443–452.