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3,5,3'-TRIIODO-L-THYRONINE INDUCES CARDIAC MYOCYTE DIFFERENTIATION BUT NOT NEURONAL DIFFERENTIATION IN P19 TERATOCARCINOMA CELLS IN A DOSE DEPENDENT MANNER

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P19 teratocarcinoma cells differentiate into neurons or muscle when treated with varying doses of retinoic acid, dimethylsulfoxide, thioguanine or butyrate. We induced cardiac differentiation in P19 cells by treating them with 3,5,3'-Triiodo-L-Thyronine (T_3). P19 cells received doses of T_3 ranging from 30 pM to 300 nM. The beating colonies were counted, and a dose response curve showed that the optimal concentration of T_3 was 30 nM. The colonies beat rhythmically for 4-6 weeks, and the cardiac myocytes showed clearly evident cardiac-specific organelles such as nexuses and atrial granules. No evidence of neuronal or skeletal muscle differentiation was seen with any of the concentrations of T_3 used. Reverse transcription-polymerase chain reaction showed these cells to express the cardiac ventricular specific marker myosin light chain 2V. T_3 is capable of inducing P19 cells to differentiate, in a dose related manner, into spontaneously beating cardiac myocytes identified as such on the basis of ultrastructural criteria. The induction of differentiation is accompanied by expression of cardiac-specific genes. These findings suggest that perhaps genes bearing thyroid response elements in their promoter regions play an important role in the cardiac differentiation induced by T_3 in P19 teratocarcinoma cells. © 1994 Academic Press, Inc.

The molecular events that result in commitment or differentiation of the myocardium in mammals are poorly understood. Although cardiac myocytes are believed to be a mesenchymal derivative, these myocytes are distinctly different from their counterparts in skeletal muscle. After the discovery of Myo D (1) it was assumed that there would be homologous factors regulating cardiac myocyte differentiation. However, it is now accepted that the members of the basic-helix-loop-helix family of DNA transcription factors that regulate skeletal muscle differentiation (2,3) do not seem to play a role in the commitment and/or differentiation of the myocardium in mammals (2). The search for specific factors involved in the differentiation of the cardiac precursors is still ongoing. Nevertheless, the study of early events in cardiac myocyte commitment and differentiation continues to be a difficult task because of the physical limitations to obtain cardiac cell precursors in early embryos. For this reason, efforts have been made to develop tissue culture systems for the study of differentiation of cardiac myocytes. The totipotential mouse

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Abbreviations: **DMSO** = dimethylsulfoxide, **MLC2V** = myosin light chain 2V, **MEM** = minimum essential medium, **PCR** = Polymerase Chain Reaction, **RA** = all-trans retinoic acid, **RAR** = Retinoic Acid Receptor, **RT-PCR** = Reverse transcription-PCR, **RXR** = Retinoid X Receptor, **T_3** = 3,5,3'-Triiodo-L-thyronine, **TBE** = Tris-Borate-EDTA buffer, **TR** = Thyroid Receptor.

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blastocyst derived embryonic ES-D3 cell line has been used to study early cardiac development (4). However, there are no known continuous cell lines that can produce cardiac myocytes (4). Another approach to this problem has involved the use of the P19 cell line which is a pluripotential, euploid, immortalized line derived from a teratocarcinoma induced in C3H/HC mice (5,6). We decided to study this cell line since it is pluripotential and can only generate a limited amount of cell lineages. Thus, the number of potentially related genes that play a role in differentiation would be *a priori* selected in favor of those genes expressed during cardiac differentiation. This may offer the advantage of having an intrinsically enriched pool of genes that play a role in the process of cardiac differentiation. The P19 cells have been shown to differentiate either into neuron or muscle phenotype depending upon induction with 1 mM RA or 1% DMSO respectively (6). Some cardiac differentiation can also be induced by low dose (1 nM) RA (5). The latter finding prompted us to investigate the potential role of T_3 in the process of cardiac differentiation in P19 cells, since the nuclear receptors of this hormone are members of the zinc finger bearing Steroid-Thyroid-Retinoic superfamily of transcription factors which are known to be morphogens and play roles in differentiation (7).

In this communication we present the results of studies showing that T_3 induces P19 cells to differentiate in a dose related manner into spontaneously beating cardiac myocytes. The induction of differentiation is accompanied by expression of cardiac specific genes. These findings suggests that target genes bearing thyroid response elements in their promoter regions play an important role in the cardiac differentiation induced by T_3 in P19 teratocarcinoma cells.

METHODS

Cell Cultures.

Cell timing and counting. P19 cells were purchased from the American Type Culture Collection (Rockville, MD) and cultured as described previously (6) except for the use of delipidized serum (see below). P19 cells were grown in suspension for 96 hrs, during which time they formed clusters or aggregates which resemble embryoid bodies from ES-D3 cells (4). Cultures were treated with the appropriate inducing compound only during this 96 hr period, unless otherwise indicated. Each treatment group was then transferred to tissue culture grade plastic flasks, and it was after attachment to the plastic that the "neuron" or "beating cardiac" differentiated phenotype was detected, usually by the 4th day. For consistency, the periods of culture in suspension are expressed in hours and subsequent periods (when the cells grew attached to the flasks) are expressed in days. The number of beating colonies and the total number of colonies per 25 mm^2 were counted at 4 days using a phase contrast inverted microscope.

Delipidized serum preparation. Since retinoids and steroid hormones may be present in the serum we employed serum that had been delipidized to eliminate endogenous lipid-soluble substances (8). The extracted protein was dried, weighed and stored at - 20°C. Treatment of 50 mL of serum by this method yielded 2 - 2.4 g of dry protein. To standardize cultures, 2.0 grams of protein were added to 500 mL of α-MEM and then the mixture was filtered through a 0.2 µm tissue culture filter. The extraction also lowered the serum concentration of T_3 from 1.8 nM to 0.2 nM (competitive immunoassay, CIBA-Corning, MA). A final concentration of 20 pM T_3 was obtained by 10 fold dilution of the serum with the tissue culture medium. For cardiac differentiation, T_3 was added to the medium in concentrations ranging from 30 pM to 300 nM. For neuronal differentiation, 1 µM RA was added to the medium.

Determination of the time of induction of differentiation. Parallel experiments were run in which the cells received treatment at only 24 and 72 hrs, or at 48 and 96 hrs. Other groups were treated only once at 24, 48, 72 or 96 hrs. All of these cultures were also counted at 4 days.

Transmission electron microscopy. Seven and 14 day old beating cultures were examined by electron microscopy. The location of beating colonies was marked on the tissue culture flasks during phase contrast examination. Subsequently the cultures were rinsed once with 10 mL of phosphate buffered saline pH 7.4 (GIBCO-BRL, MD) and fixed for 4 hrs with 1% glutaraldehyde-4% formaldehyde in phosphate buffer pH 7.2 (9) pre-

warmed to room temperature. The marked colonies were dissected, transferred to vials, fixed with 1% osmium tetroxide in 0.1 M sodium cacodylate buffer pH 7.3, for 1 hr and then flat embedded in Epon-Araldite or Polybed 812. One μm thick sections perpendicular to the surface of the colonies were cut, stained with alkaline toluidine blue and examined with a light microscope to select areas for electron microscopic studies. Ultrathin sections were stained with uranyl acetate and lead citrate (9) and examined by transmission electron microscopy.

RT-PCR. Total RNA was extracted using RNAzol (Biotecx, TX) according to the manufacturer's instructions. Oligo-$(dT)_{25}$-Dynabeads (Dynal, Great Neck, NY) were used for mRNA purification. mRNA (2-5 μg) were digested with 2 units of DNAse RQ1 (Promega, Madison, WI) for 30 minutes to eliminate genomic DNA. Subsequently cDNA was synthesized with 20 units of SuperScript II reverse transcriptase (GIBCO-BRL, Gaithersburg, MD) in the manufacturer's supplied buffer at 37°C for 1 hour. Primers for PCR were synthesized in an oligonucleotide synthesizer (ABI-PCR-Mate, Foster City, CA) and purified using thin layer chromatography (Sure-Pure, USB, Cleveland, OH). To perform the PCR, we used the primer sequences and cycling conditions reported by Miller-Hance et al. (4). The targets chosen were MLC2V (499 bp) and β-Tubulin (317 bp). We designed 20-mer oligonucleotide probes for radioactive detection of PCR product. The sequence for the MLC2V probe was 5'-AAGGGGCTGATCCTGAAGAG-3' and for the β-Tubulin probe 5'-ACGAGCAGATGCTGA-ACGTG-3'. PCR's were run in a Perkin Elmer 9600 thermocycler using "hot start" (10). The reactions were run in a volume of 25 μL as previously described (11). The control samples included: 1) negative control, master PCR mix without any target DNA; and 2) positive control, pooled cDNA from mouse embryo and adult heart. Five picomoles of each probe were end-labeled with [γ-^{32}P]ATP using 5 units of T4 polynucleotide kinase according to the manufacturer's instructions (Promega, WI). Analysis was done in 5 % non-denaturing polyacrylamide gels at 100 volts in 1 X TBE buffer (12). The gels were then dried and autoradiographed overnight.

RESULTS

T_3-induced differentiation of P19 cells. The dose response curve obtained is shown in **Figure 1.** The percentages of beating cells were; 9.7, 17.9, 21.9 and 16.9 % at 30 pM, 300 pM, 30 nM and 300 nM T_3 respectively ; DMSO (1%) induced 9.36% beating colonies. (All the values represent the mean from 2 separate experiments). An unexpected feature of our delipidized cell culture method was the great longevity of the beating cardiac phenotype which consistently lasted 4 - 6 weeks as opposed to the previously reported figure of 3-4 days (6). The experiments in which treatment was carried out only on specific days or on alternate days allowed us to determine that, in the presence of T_3, a major event that irreversibly triggers the program of cardiac differentiation occurs between 24 and 48 hrs in suspension culture. Differentiation does not occur unless the cells are treated during the first 48 hours. Interruption of treatment after 48 hrs does not prevent differentiation.

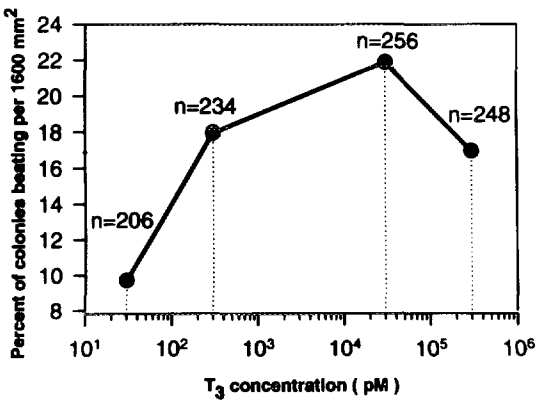

Figure 1. **Dose response curve of T_3-mediated cardiac differentiation in P19 cells.** For each dose of T_3 the number of colonies counted (n) is shown. The colonies present in 64 squares (25 mm^2 each) were counted. The actual beating percentages were: 30 pM = 9.70%; 300 pM T_3 = 17.94%; 30 nM T_3 = 21.87%; and 300 nM T_3 = 16.93%.

Phase contrast microscopy. Beating usually began in small colonies which eventually enlarge and/or coalesce to clearly form larger colonies measuring up to 3 mm in diameter. These large colonies of beating myocytes often show one or two prominent foci of 4 to 10 cells acting as a pacemaker for the colony on phase contrast microscopy. Branching colonies with long tubular and fusiform beating cells were also present. These morphologies persisted in long term culture, during which the colonies became larger and beat more consistently. The longevity of the culture was perhaps limited by the dense network of collagen laid down by these cells. This network appeared to shrink, thus inducing detachment of the colonies from the culture flask.

Ultrastructural examination. Semithin (1 μm) sections perpendicular to the surface of the colonies consistently showed three distinct strata. The uppermost stratum always consisted of a single layer of flat undifferentiated cells with abundant surface microvilli. The myocytes were located in the middle stratum. The lower stratum always showed undifferentiated mesenchymal cells in contact with the plastic surface of the tissue culture flask. Ultrastructural study showed cardiac myocytes in various stages of maturation. At 7 days, the myocytes showed single central nuclei, abundant rough-surfaced endoplasmic reticulum, glycogen granules and numerous thick and thin filaments in various stages of organization. The areas of sarcomere formation were present as subsarcolemmal deposits of Z-band-like material with myofibrillar insertion sites showing thick and thin filaments. In cross section, these sarcomeres showed the typical hexagonal array of thick filaments surrounded by thin filaments (**Figure 2A** and **2B**). Small numbers of tiny nexuses were seen connecting finger-like projections of adjacent myocytes (**Figure 2B**, arrowheads). Zonulae adherentes also formed intercellular connections at this time (**Figure 2C**, arrowheads). At 14 days, the myocytes showed central nuclei and abundant mature sarcomeres (**Figure 2D**). The thick filaments measured approximately 15 nm in diameter and 800 nm in length, and the thin filaments measured approximately 8 nm in diameter and 500 nm in length. Sarcomeres at these stages measured an average of 1.7 μm in length. Occasional myocytes also contained atrial granules (**Figure 2E**) (13). At this stage no clear T-tubular system was found. Typical intercalated discs with nexuses (**Figure 2F**) and desmosomes (**Figure 2G**) connecting adjacent cardiac myocytes were easy to find (13). Intercellular junctions did not connect cardiac myocytes with the surrounding undifferentiated mesenchymal cells. In 7 and 14 day old, differentiated cultures, extensive search failed to reveal any ultrastructural features of skeletal muscle phenotype.

Reverse transcription-polymerase chain reaction (RT-PCR). The results of the RT-PCR are shown in **Figure 3**. Amplification of MLC2V was first evident at 48 hrs and throughout the culture time intervals studied as well as in the mouse heart control. Amplification of β-Tubulin was also positive in the positive controls as well as all stages of P19 cell differentiation studied.

DISCUSSION

We studied the effects of T_3 rather than those of the non-physiologic, non-specific inducer DMSO because: 1) we hypothesized that T_3 may have an effect on the differentiation of P19 cells since it is a member of the Steroid-Thyroid-Retinoid superfamily of transcription factors as is RA, and 2) T_3 is a physiological substance that regulates cardiac genes (14,15) and induces hypertrophy in neonatal (16) and adult animals (17). Furthermore, since neither induction of cardiac differentiation of P19 cells by T_3 nor a role of T_3 in the early embryonic heart have been reported (18), we wanted to explore this possibility in a cell line that hypothetically may share common events with normal embryonic cardiac development.

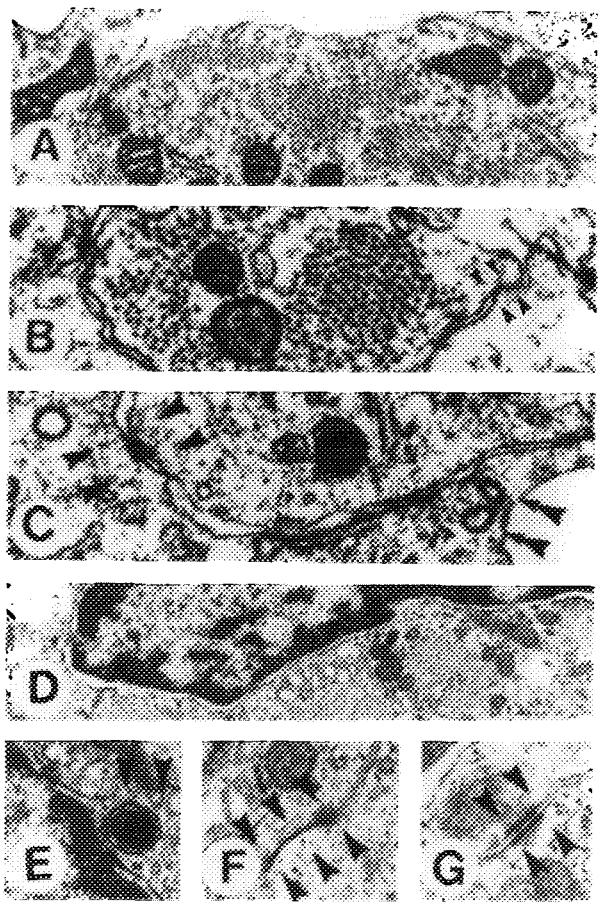

Figure 2. **Transmission electron micrographs of beating P19 cells.** (**A**) At 7 days, P19 cells show scant but organizing sarcomeres (X 10,000). (**B**) The sarcomeres show the typical 6:1 arrangement of actin : myosin filaments. Tiny nexuses were present connecting adjacent myocytes (arrowheads)(X 15,000). (**C**) They also show immature intercalated discs with early zonulae adherentes formation (arrowheads) and abundant rough endoplasmic reticulum (2 arrows). (X 10,000).(**D**) At 14 days the sarcomeres are more abundant. (X 8,000). (**E**) There are cells with typical membrane bound-dense core-atrial granules (X 20,000). The intercalated discs show nexuses (**F**) (X 20,000) and desmosomes (**G**) (X 20,000).

In our study, the use of delipidized serum provided a milieu in which we could carry out tissue culture studies without interference of retinoids or steroids and virtually no T_3. The percentage of beating colonies in the T_3-treated cultures was much higher (21.87 %) than that (maximum of 9.36%) observed in cultures treated with DMSO and grown with delipidized serum. The ultrastructural documentation of sarcomeres and specialized components of the intercellular junctions (desmosomes and nexuses) in the cells of beating colonies established the cardiac nature of the differentiating cells (13). The presence of nexuses explains the coordinated beating of the colonies of myocytes. The finding of atrial type granules in some of the beating cells is also new information that provides additional proof of the cardiac nature of the differentiated cells (13). It is important to note that when treated with a wide range of concentrations of T_3, P19 cells only differentiate into cardiac phenotype. We did not find neuronal differentiation of P19 cells at any dose level of T_3, in contrast to the findings with RA, which induces cardiac

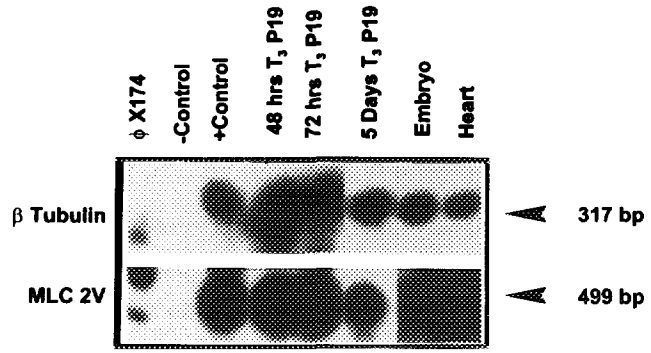

Figure 3. **RT-PCR of β-Tubulin and MLC2V.** Lane 1 = Size marker ϕX174 Hae III restricted. Lane 2 = Negative (no template) control. Lane 3 = pooled cDNA control. Lane 4 = 48 hrs. "cardiac" cDNA, Lane 5 = 72 hrs. "cardiac" cDNA. Lane 6 = 5 day "cardiac" cDNA. Lane 7= mouse embryo cDNA. Lane 8 = Adult mouse heart cDNA.

differentiation at low doses and neuronal differentiation at higher doses (5,6). Furthermore, although microscopic observations of P19 cells differentiated with DMSO and RA suggested that beating cells transform into skeletal muscle cells as a function of time (5,6), we did not observe ultrastructural evidence of differentiation of T_3-treated cells into skeletal muscle cells at 7 or 14 days.

The MLC2V RT-PCR target is an mRNA transcript which is expressed during cardiac development and has been used as a marker of ventricular specification in embryoid bodies(4). The detection (by RT-PCR) of the expression of this transcript under conditions identical to those employed for detecting the MLC2V transcript (4) after T_3 induced differentiation of P19 cells indicates that at least some of the beating cells may have a ventricular phenotype. We determined that administration of T_3 during the first 48 hr in suspension culture triggers the program of cardiac differentiation in P19 cells. This event is judged to be irreversible since withdrawal of T_3 after 48 hrs did not prevent cardiac differentiation. This was confirmed in cultures treated on alternate or only on specific days with T_3 as long as the treatment was given during the first 48 hrs in culture. Recognition of this sequence of events may allow use of this model for investigation of the potential role of transcription factors of the Steroid-Thyroid-Retinoic superfamily as potential mediators of cardiac differentiation.

This superfamily of transcription factors belongs to the class of zinc finger proteins which have domains with well defined functions for binding to ligands, DNA, and other receptors in order to form dimers (19-22). The consensus sequence RGG(T/A)CA in the promoter region of genes regulated by these transcription factors is recognized by TR and is common to other members of the superfamily. The specificity of recognition is dictated by: 1) the number of repeats of the consensus; 2) the 5' to 3' orientation of the repeats and; 3) the spacing between repeats (4 base pairs for TR's (23), 5 for RAR's (23), 3 or 6 for Vitamin D_3 receptors (24)). T_3 exerts its action through two TR (19) which can be active as homodimers as well as heterodimers, i.e., in combination with RAR (22), RXR (22), VDR (24) and other receptors (21,22). Regulation of RXR by T_3 in the heart has also been shown (25).

This finding is of interest since it proves that T_3 can regulate the levels of RXR in the heart and thus alter gene expression. Similar mechanisms of control of expression of TR or RXR may play a role during cardiac embryonic differentiation. In fact, very recently it has been shown that a "knock out" of the RXRα gene in mice produced severe deficiency of the formation of the ventricular walls (26) but no studies have been performed that focus on the role of T_3 in the early stages of cardiac differentiation.

Thus, we present a model of cardiac myocyte differentiation induced by T_3 in P19 teratocarcinoma cells that produces long lived beating colonies in which genes bearing with response elements in their promoter regions recognized by TR/TR or TR/RXR dimers may play an important role in the development of the cardiac phenotype. The model may be useful in investigations of the role of diverse transcription factors in the process of cardiac differentiation.

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