Triiodothyronine Induces Over-Expression of α-Smooth Muscle Actin, Restricts Myofibrillar Expansion and Is Permissive for the Action of Basic Fibroblast Growth Factor and Insulin-like Growth Factor I in Adult Rat Cardiomyocytes

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Abstract

Effects of triiodothyronine (T3) on the expression of cytoskeletal and myofibrillar proteins in adult rat cardiomyocytes (ARC) were followed during two weeks of culture in the presence of 20% T3-depleted (stripped) FCS. Control cultures expressed mainly β-myosin heavy chain (MHC) mRNA. T3 caused a switch to α-MHC expression and a dose-dependent increase of α-smooth muscle (α-sm) actin mRNA and protein. In parallel, the number of α-sm actin immunoreactive cells increased from 1% in controls to 29 and 62% in ARC treated with 5 and 100 nM T3. In the presence of T3, cells exhibited a higher beating rate than controls. The distribution of myofibrils in T3-treated cells was restricted to the perinuclear area with a sharp boundary. Only 5% of the control cells but 30 and 62% of the T3-treated (5 and 100 nM) ARC showed this restricted myofibrillar phenotype. Basic fibroblast growth factor (bFGF) which restricts myofibrillar growth and upregulates α-sm actin in ARC cultured with normal FCS had no effect on α-sm actin in ARC cultured in stripped FCS, but potentiated the effect of T3. In contrast, insulin-like growth factor I (IGF I), which suppresses α-sm actin and stimulates myofibrillogenesis in the presence of normal FCS suppressed T3-induced α-sm actin expression in stripped FCS. Thus, T3 appears to be permissive for the action of bFGF and IGF I on α-sm actin expression. (*J. Clin. Invest.* 1996. 98:1737–1744.) Key words: myocardium-cytology • myofibrils • cardiac-proteins • actin-isoforms • gene-expression

Introduction

Patients with hyperthyroidism and hypothyroidism frequently develop cardiovascular disorders. Cardiac performance can be directly correlated with the thyroid hormone serum levels in patients (1). In hyperthyroidism, heart rate, contractility, ejection rate, and coronary blood flow are all increased while the systemic vascular resistance is concomitantly reduced (2). These changes are accompanied by cardiac hypertrophy and may result in heart failure (3). On the other hand, thyroid hormone deficiency is associated with decreased contractility and with cardiac dilation (4, 5). Both types of changes can be reversed by redressing the unbalanced thyroid hormone status (6, 7).

Thyroid hormones exert direct and indirect effects on the heart (for review see reference 8). The indirect effects seem to be mediated mainly by up- or downregulation of α- and β-adrenergic receptors in laboratory animals as well as in patients (9, 10). Thus, the positive chronotropic and inotropic effects induced by the biologically active thyroid hormone triiodothyronine (T3)[1] in the isolated rat heart model can be abolished by β-adrenergic receptor blockade (11). The direct effects of T3 result from its binding to nuclear receptors which function as transcription factors (12). The T3-induced increase of cardiac output and the RNA/DNA ratio as well as the development of hypertrophy are not affected by β-blockade (11, 13).

During heart hypertrophy due to cardiac overload, a number of fetal proteins are reexpressed, whereas others which are specific for the fully differentiated adult phenotype are downregulated (14–16). The hypertrophic stimulus induces a rapid transient expression of immediate early genes, e.g., c-*fos*, c-*jun*, egr-1. This is followed by reexpression of proteins normally occurring only in the fetal ventricle, such as atrial natriuretic factor, β-myosin heavy chain (β-MHC), atrial myosin light chain-1, α-skeletal muscle actin, α-smooth muscle actin (α-sm actin), fetal troponin-T isoforms, β-tropomyosin and also other proteins neither connected with cytoskeletal nor with contractile structures. At the same time α-MHC is downregulated.

In cultured cardiomyocytes two types of triggers lead to the hypertrophic phenotype (17): mechanical triggers like cell deformation, and trophic triggers such as α1-adrenergic agonists, vasoactive peptides (endothelin-1 and angiotensin II), growth factors (in particular bFGF, IGF I, and transforming growth factor-β), adrenocorticoids, insulin, growth hormone, and thyroid hormones.

T3 upregulates α-MHC and downregulates β-MHC in small laboratory animals in vivo (18) as well as in primary cultures of cardiomyocytes (19). In larger mammals including man, β-MHC is the main myosin heavy chain isoform under normal conditions (20). Nevertheless, it has been observed that levothyroxine treatment of a patient with hypothyroidism

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1. *Abbreviations used in this paper:* α-sm actin, α-smooth muscle actin; ARC, adult rat cardiomyocytes; bFGF, basic fibroblast growth factor; MHC, myosin heavy chain; T3, triiodothyronine; T4, thyroxine.

and dilated cardiomyopathy increased the formerly low α-MHC mRNA level (21). Thus, the effect of thyroid hormones on MHC isoforms is opposite to that of cardiac overload.

Adult rat cardiomyocytes (ARC) in culture lend themselves to test the effects of single factors in a controlled way. bFGF has recently been shown to induce a large increase in α-sm actin protein accumulation in the cytoskeleton of ARC, which coincides with inhibition of myofibrillar growth, so that the cross-striated sarcomere structures remain restrained to the central cell region (22). In contrast to bFGF, IGF I promotes myofibrillar growth and suppresses accumulation of α-sm actin in ARC in culture (22, 23). We therefore tested whether T3 also affects the expression of α-sm actin and whether this may also be coupled to changes in the cytoskeletal-myofibrillar interrelation. Furthermore, it was of interest to see whether the effects of bFGF and IGF I are linked to the action of T3 or whether they operate independently of T3.

Methods

Cell culture. Ventricular cardiac muscle cells of adult female rats (Sprague-Dawley-Ivanovas, 2-mo-old) were isolated by retrograde perfusion of hearts with collagenase type 2 (Worthington Biochemical Corporation, Freehold, NJ) according to (24). After perfusion, the heart tissue was minced and incubated at 37°C for another 10 min in KB medium (25) containing collagenase. Cells were cultured in dishes coated with 1% gelatine in M-199 supplemented with 20% stripped FCS (GIBCO BRL, Paisley, UK), 1% penicillin/streptomycin, 20 mM creatine. To inhibit growth of contaminating cells, 10 μM 1-β-D-arabinofuranosyl-cytosine was added throughout the culture period. The medium was changed after 2, 7, and 12 d. T3 (Sigma Chemical Co., St. Louis MO), recombinant human bFGF (Boehringer Mannheim, Mannheim, Germany) and recombinant human IGF I (Ciba-Geigy, Basel, Switzerland) were added at the indicated concentrations after the first change of the medium on day 2.

Depletion of T3 and T4 from FCS (stripped FCS). Serum was incubated at room temperature with 50 mg/ml AG 1-X8 resin (200–400 mesh, chloride form, BioRad, Richmond, CA) which had been previously washed three times with distilled water according to (26). After 5 h of incubation under stirring, the resin was removed by centrifugation at 1,000 *g* for 10 min. An additional quantity of fresh resin was added at a concentration of 50 mg/ml to the residual serum, and the serum was incubated for additional 15–18 h at room temperature. The resin was finally removed by two centrifugations at 1,000 *g* for 10 min and at 30,000 *g* for 20 min at room temperature. The serum was sterilized by filtration through a 0.2-μm filter (cellulose acetate type Falcon 7111; Becton Dickinson, Lincoln Park, NJ) and stored at −20°C. The concentrations of total and free T3 and thyroxine (T4) in normal and stripped FCS were determined by a chemiluminescence immunoassay at the Institute for Clinical Chemistry of the University Hospital of Zurich.

RNA isolation and Northern blotting. Total RNA was isolated according to standard procedures (27). Cells were washed three times with ice-cold PBS, directly lysed in 2.5 ml of 4 M guanidinium isothiocyanate containing 5 mM sodium citrate, pH 7.0, 0.1 M β-mercaptoethanol and 0.5% sarcosine, and centrifuged through a CsCl gradient. The protein-containing supernatant was kept for Western blot analysis. RNA was dissolved in diethylpyrocarbonate-treated H_2O and concentrations were determined spectrophotometrically. Denatured RNA (2–10 μg) was electrophoresed on a 1% agarose gel containing 2 M formaldehyde, transferred onto a nylon membrane (Hybond-N; Amersham, Aylesbury, UK) and fixed by UV crosslinking. Filters were prehybridized for 2 h at 42°C in a solution containing 50% formamide (25% for α-MHC oligo probe), 5× Denhardt's [100× = 2% (wt/vol) Ficoll and 2% (wt/vol) polyvinyl pyrrolidone], 5× SSPE (20× = 3.6 M sodium chloride, 0.2 M sodium phosphate and 0.02 M EDTA, pH 7.7), 0.2% SDS and 100 μg/ml of heat denatured salmon sperm DNA. The following cDNAs and oligonucleotides were used for hybridization: α-smooth muscle actin cDNA (kindly provided by Dr. R. S. Blank, University of Virginia); yeast 18 S cDNA (from Dr. M. Kalousek, University Hospital Zurich, Switzerland) and oligonucleotides (20-mers, synthetized by Microsynth, Balgach, Switzerland) specific for α- and β-MHC RNAs (28). cDNA probes were labeled by random primer extension using a commercial kit (Boehringer Mannheim) and [α-^{32}P]deoxycytidine 5′-triphosphate (3,000 Ci/mmol, Amersham). Oligonucleotide probes were labeled with [γ-^{32}P]adenosine 5′-triphosphate (5,000 Ci/mmol, Amersham) using a 5′ end labeling kit (Boehringer Mannheim).

Hybridization was performed at 42°C (37°C for α-MHC) in the same solution as described for prehybridization. After 18 h of hybridization with the oligo probes, filters were washed twice for 15 min at room temperature with 2 × SSC, 0.1% SDS and subsequently three times in the same buffer for 20 min at 39°C (α-MHC) or 42°C (β-MHC). After 48 h of hybridization with the cDNA probes, the filters were washed twice for 15 min at room temperature with 0.1× SSC, 0.1% SDS and subsequently three times in the same buffer for 20 min at 55°C (α-sm actin cDNA) or at 65°C (18 S cDNA). mRNA levels were quantified by scanning densitometry using a BioRad video densitometer. Variations of gel loading were corrected against the corresponding 18 S ribosomal RNA values.

Western blotting. After centrifugation of the RNA through the CsCl gradient, the upper phase containing the protein fraction was desalted by centrifugation over a BioGel P-6DG (Bio-Rad) spun column equilibrated in 0.1 M ammonium bicarbonate. The eluate was lyophilized, dissolved in 100 μl H_2O, and the protein concentration was determined using the BCA protein assay (Pierce, Rockford, IL). 5 μg of total protein was separated on a 10% SDS polyacrylamide minigel (Hoefer Scientifics, San Fernando, CA) and transferred to nitrocellulose using a mini transfer cell (BioRad). α-sm actin was detected with an α-sm actin specific antibody (Sigma Chemical Co., mouse IgG2a clone 1A4, Nr. A2547) and the enhanced chemiluminescence (ECL) detection system (Amersham, RPN 2106). The blots were stripped in 100 mM β-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl pH 6.7 at 50°C for 30 min and reprobed with an antibody specific for all actin isoforms (ICN Biochemicals, Costa Mesa, CA; mouse anti-actin clone C4). Protein levels were quantified by scanning densitometry using a BioRad video densitometer.

Immunocytochemistry. Cells were fixed and permeabilized as described (23). F-actin was stained by rhodamine-phalloidin (Molecular Probes, Inc., Eugene, OR). The following antibodies were used for immunostaining: a mAb against myomesin, a sarcomeric M-line protein (29); a mAb against α-sm actin (Sigma Chemical Co., clone 1A4), a mAb against panmyosin (Amersham, mouse IgM RPN 1169) and a polyclonal antiserum against cardiac C-protein (30). FITC-conjugated antibodies (goat anti–mouse: Pierce, Rockford IL; and goat anti–rabbit: Cappel Research Reagents, Organon Teknika N.V., Turnhout, Belgium) as well as Texas red–conjugated antibodies (donkey anti–mouse; Jackson Immuno Research Lab. Inc., Milan Analytica AG, La Roche, Switzerland) were used as secondary antibodies.

Microscopy. Immunofluorescence microscopy was performed with a Zeiss Fluorescence Microscope with a Neofluar × 40/0.75 objective lens (Carl Zeiss Co., Oberkochen, Germany). Fluorescence images were produced by a confocal laser scanning microscope (Zeiss Axiophot fluorescence microscope) with a Zeiss Neofluar × 40/1.3 objective lens connected to a Bio-Rad MRC-600 confocal scanner (BioRad, Lasership Ltd., Oxfordshire, UK) and a Silicon Graphics Personal Iris 4D/25 Work-station (Silicon Graphics, Mountain View, CA).

The number of α-sm actin positive cells was determined by counting immunoreactive cells in different cultures after 12–13 d. Myofibrillar restriction was determined by counting cells with myofibrillar structures forming a sharp boundary and filling < 50% of the cell area. Only cells with an intact cytoskeleton as visualized by phalloidin-rhodamine staining and with two nuclei and a clear myofibrillar pattern were counted.

Table I. Influence of Resin-treatment on the Level of Total and Free T3 and T4 in FCS

	Control FCS		Resin-treated FCS	
	100%	20%	100%	20%
Total T3 (nM)	2.4	0.6	0.3	< 0.3
Total T4 (nM)	306	37	107	< 7
Free T3 (pM)	5.4	2.6	< 1.7	< 1.7
Free T4 (pM)	24.5	13.8	7.2	2.6

Total and free T3 and T4 were determined by a chemiluminescence immunoassay. Total T3 and T4 in 20% resin-treated FCS and free T3 in 100 and 20% resin-treated FCS were below the limit of detection. The error of this method is below 5%.

To determine the beating rates of ARC, the cultures were maintained at 37°C in a humidified chamber containing 5% CO_2 which was connected to a microscope equipped with a video screen. Cells were observed for 10 min in three different experiments each.

Statistics. The results are expressed as mean±SD. Student's *t* test was used for evaluation of significance. Values of $P < 0.05$ are considered to be significant.

Results

Effects of T3- and T4-depleted fetal calf serum. ARC in long-term culture undergo extensive morphological changes. Most of the freshly isolated rod-shaped ARC round up before they attach to the substratum (31, 32). Over the period of 2 wk, they grow considerably in size, form pseudopodia in all directions, make contact with neighboring cells and start beating. This complex hypertrophic reaction requires the presence and interaction of several cell mediators and growth factors which are not yet known. Normal FCS contains considerable amounts of T3 and T4 (Table I). Therefore, it was depleted of T3 and T4 before the experiments (Table I). Total and free T3 concentrations in 20% stripped FCS were below the limit of detection.

After 2 wk in culture, control cells in 20% stripped FCS exhibited mainly polygonal cell shapes with fewer pseudopodia-like structures than cells kept in untreated FCS. After treatment with T3, cells displayed more pseudopodia and star-like cell shapes and were beating vigorously.

Control ARC in long-term culture change their MHC isoform expression from the α- to the β-isoform (31). T3 is known to induce the expression of the α-MHC isoform (19, 28, 33). The effect of stripped FCS on MHC isoform expression is shown in Fig. 1. ARC cultured for 16 d in the presence of 20% untreated FCS expressed predominantly β-MHC mRNA and low levels of α-MHC mRNA. In the presence of stripped serum, ARC expressed no α-MHC mRNA, but high levels of β-MHC mRNA. Addition of increasing amounts of T3 induced α-MHC mRNA and suppressed β-MHC mRNA. It is known that α-sm actin which is fetally expressed becomes reexpressed transiently in ARC cultured with normal FCS (23, 24). Also in this case, the transcription of α-sm actin mRNA, like that of α-MHC mRNA, is dependent on the presence of T3 in FCS (Fig. 1). The effects of 1 nM T3 in stripped serum corresponded to those observed with untreated serum.

Effects of T3 on α-sm actin mRNA expression and protein accumulation. In freshly isolated rod-shaped cells, α-sm actin is not expressed, but it appears during the "re-differentiation" in vitro reaching its maximum after 2 wk (22–24). T3 caused a dramatic increase of α-sm actin mRNA (Fig. 2, *left panel*) which was dose-dependent: treatment with 1, 10, and 100 nM T3 resulted in 231, 590, and 709% of the control α-sm actin mRNA expression (Table II). A significant increase in α-sm actin protein accumulation occurred already with 0.3 and 1 nM T3 and tended to level 5–6-fold above control with 3–10 nM T3 (Fig. 2 and Table II). Thus, both message and protein accumu-

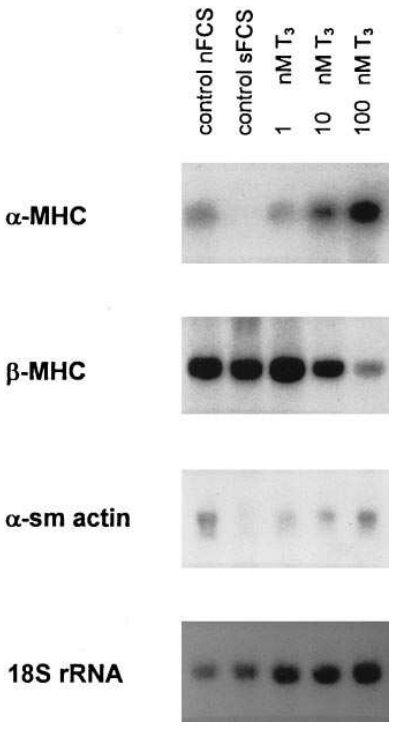

Figure 1. T3-dependent regulation of α- and β-MHC mRNA and α-sm actin mRNA expression in ARC. Total RNA was isolated from 16-d-old ARC and analyzed by Northern blotting. One representative experiment out of five is shown. Variability of gel loading was checked by hybridization with a 18S rRNA specific probe (control nFCS, control ARC cultured in 20% unstripped FCS; control sFCS, control ARC cultured in 20% stripped FCS).

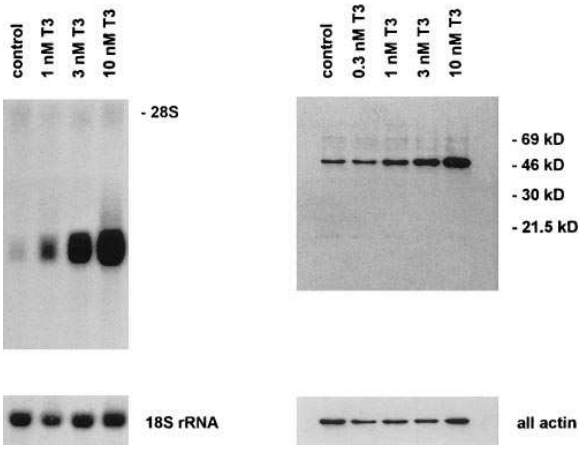

Figure 2. T3-dependent stimulation of α-sm actin mRNA and protein levels. Total RNA (4 μg) isolated from 12-d-old ARC was analyzed by Northern blotting (*left panel*). Variability of gel loading was controlled by hybridization with a 18S rRNA specific probe. The protein fractions from the same samples were desalted, concentrated and 5 μg of total protein was subjected to Western blotting (*right panel*). The blot was reprobed with an antibody reacting with all actin isoforms.

Table II. α-sm Actin mRNA and Protein Expression in 12–14 d old ARC Cultures

Treatment	α-sm actin mRNA levels	Significance versus controls	Number of experiments	α-sm actin protein levels	Significance versus controls	Number of experiments
Controls	100		6	100		3
0.3 nM T3	n.d.			168±7.6	$P < 0.005$	3
1 nM T3	231±51	$P < 0.005$	5	319±65.9	$P < 0.05$	3
3 nM T3	n.d.			528±65.9	$P < 0.01$	3
10 nM T3	590±200*	$P < 0.01$	5	596±71.3	$P < 0.01$	3
100 nM T3	709±263‡	$P < 0.005$	6	n.d.		

OD-values of the 2.1-kb α-sm actin mRNA bands and of the 42-kD α-sm actin protein bands were measured by scanning densitometry. RNA data were normalized against 18S rRNA values. Controls are defined as 100%, relative values are given in percents±standard deviations. *$P < 0.01$ versus ARC treated with 1 nM T3; ‡$P < 0.05$ versus ARC treated with 10 nM T3.

lation responded positively to small concentrations of T3 in parallel. Both α-sm actin mRNA and protein expression reached maximal values as early as 6 d of T3-treatment (not shown). The increase of α-sm actin mRNA and protein by T3 was confirmed by immunocytochemistry. The number of cells staining for α-sm actin was 1% in control cultures, but increased to 29 and 62% in ARC treated with 5 and 100 nM T3 (Table III).

Effects of T3 on contractile and cytoskeletal structures. After the attachment of the cells to the substratum, the myofibrillar apparatus of ARC "degenerates" before new myofibrillar structures develop and grow out from the perinuclear region into the cell periphery (31, 32). The cytoskeletal and contractile structures of ARC were visualized by double staining for F-actin and for myomesin as a myofibrillar marker (Fig. 3). Without T3, myofibrils follow the well-developed cytoskeletal structures into the periphery. After 2 wk in culture they usually fill almost the entire cell area (Fig. 3 *A*).

In contrast, with T3 the myofibrillar structures become restricted to the perinuclear region and do not expand into the periphery (Fig. 3, *B* and *C*). The restricted myofibrils still seem to be following the actin cables but stop abruptly by forming a sharp boundary against the peripheral actin cytoskeleton. After the initial "degeneration," reformation of myofibrillar structures starts in the central cell region within 3–5 d. Under T3 treatment the boundary where myofibrils stop growing out, develops in the following days. This phenotype persists from around day 10 as long as the cells remain alive (2–3 wk). After 2 wk, the percentage of cells with a restricted myofibrillar pattern was 5% in control ARC and increased to 30 and 62% in ARC treated with 5 and 100 nM T3, respectively.

Double staining for α-sm actin and cardiac C-protein as a sarcomeric marker revealed that the α-sm actin is deposited around the perinuclear area and follows the stress fibre-like structures into the periphery (Fig. 4 *A*). Within the central region, staining was almost absent and no structures were detectable. The shape of the myofibrillar area staining for cardiac C-protein (Fig. 4 *B*) mirrors exactly that of the region left unstained by α-sm actin (Fig. 4 *A*). Thus, the boundary of the restricted myofibrils coincides with that of α-sm actin emanating from there into the periphery. Confocal microscopic sectioning in 0.3-μm intervals indicated that the two structures are preserved in complementary shape throughout the cell (not shown).

Myosin content was examined by double staining for F-actin and with a monoclonal antibody reacting with all MHC isoforms (pan-myosin). Without T3-treatment both myosin and actin staining showed cross-striation throughout the entire cell (Fig. 5, *C* and *D*). In contrast, in T3-treated cells the actin staining pattern displayed cross-striation in the central region of the cell (Fig. 5 *A*). Similarly, myosin staining exhibited cross-striation only in the central area while outside of that area the myosin was not arranged in sarcomeres and gave a diffuse staining (Fig. 5 *B*).

Table III. Number of α-sm Actin Immunoreactive Cells and of Cells with Restricted Myofibrils in 12–14-d-old ARC Cultures

Treatment	Controls	T3 (5 nM)	T3 (100 nM)	T3 + bFGF	bFGF	IGF I
α-sm actin immunoreactive cells (%)	1.09±1.74	28.7±18.4	61.6±13.8*	74.1±15.5	2.08±2.78‡	0
Number of cells counted	963	618	1070	440	547	240
(number of experiments)	(8)	(4)	(8)	(5)	(5)	(3)
Significance level versus controls		$P < 0.05$	$P < 0.001$	$P < 0.001$	n.s.	n.s.
Cells with restricted myofibrils (%)	5.03±7.65	30.3±18.2	61.7±17.3	53.0±10.1	26.9±24.0*	4.73±5.33
Number of cells counted	669	252	770	414	436	252
(number of experiments)	(7)	(3)	(7)	(5)	(5)	(3)
Significance level versus controls		$P = 0.09$	$P < 0.001$	$P < 0.001$	$P < 0.05$	n.s.

Cells were counted in ARC cultures treated with 5 and 100 nM T3, 50 nM IGF I, 1.4 nM bFGF, 100 nM T3 plus 1.4 nM bFGF in stripped FCS and non-treated controls (±standard deviations). *$P < 0.05$ and ‡$P < 0.001$ versus ARC treated with T3 plus bFGF; n.s., not significant.

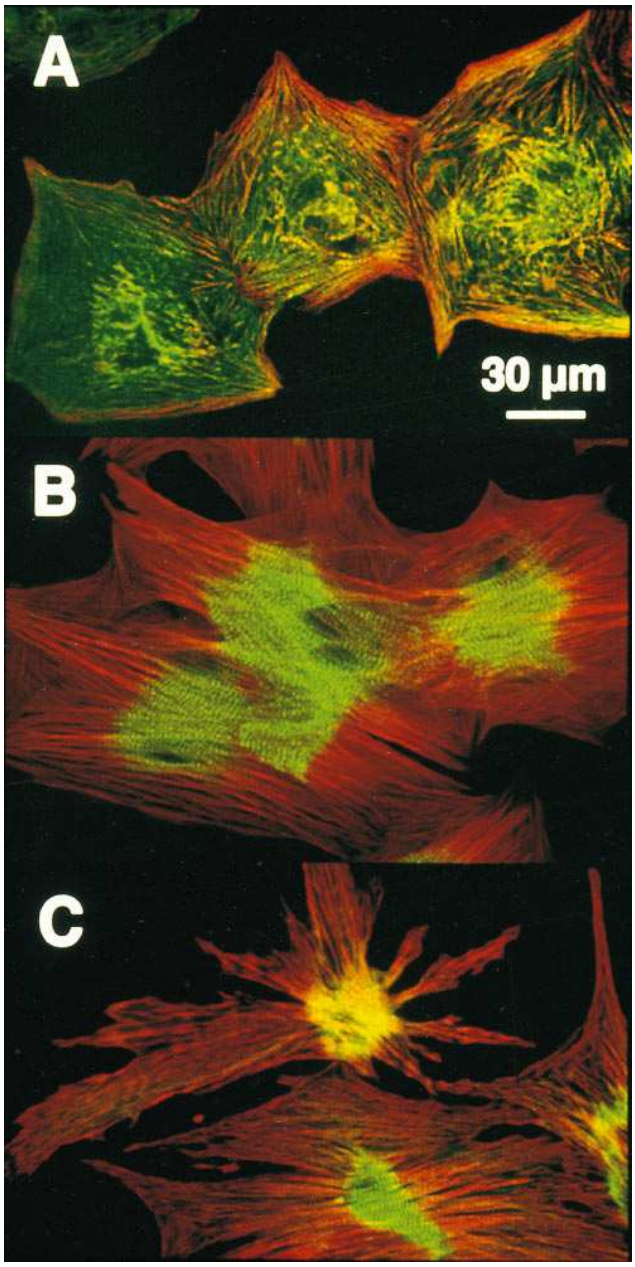

Figure 3. Confocal micrographs (layer at 0.3 µm from bottom) of adult rat cardiomyocytes after 12 d in culture double-labeled with phalloidin-rhodamine for F-actin (*red*) and with a monoclonal antibody against myomesin (*green-yellow*). *A*, control; *B*, 5 nM T3; *C*, 100 nM T3.

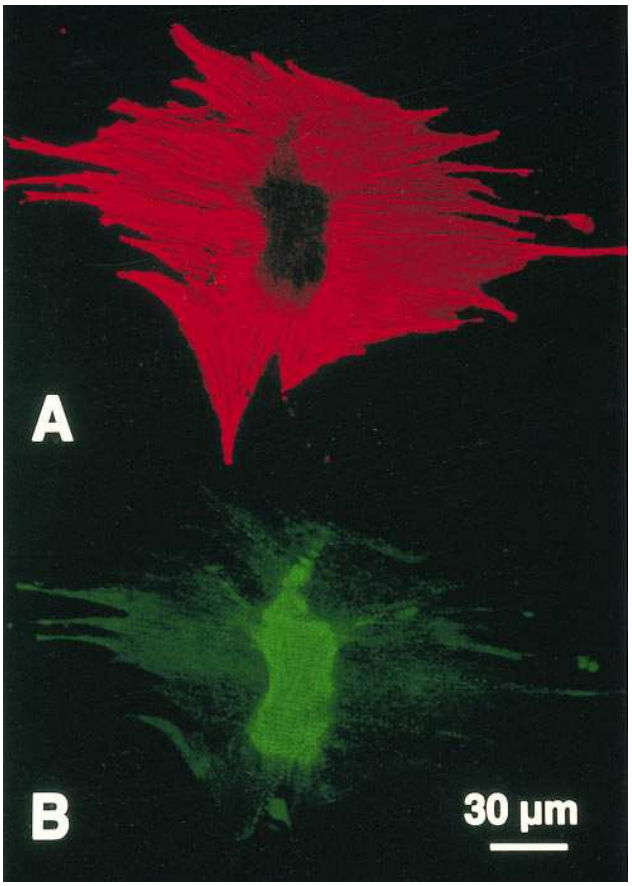

Figure 4. Confocal micrographs (layer at 0.3 µm from bottom) of adult rat cardiomyocytes treated for 12 d with 100 nM T3. Double labeling with a monoclonal antibody against α-sm actin (*A*, *red*) and cardiac C-protein antiserum (*B*, *green*).

Despite their restricted sarcomeric pattern, T3-treated ARC were beating vigorously. The average beating rate of 11-d-old control cells was 47±37 (n = 37) beats per minute. In the presence of 2, 10, and 100 nM T3 beating rates increased to 92±53 (n = 22), 141±32 (n = 32) and 132±15 (n = 38) per minute, respectively. Although the variability from cell to cell was high in all experimental groups, the increase of the beating rate with 10 and 100 nM T3 was highly significant ($P < 0.001$) versus controls.

Permissive effects of T3 on bFGF and IGF I action. For experiments in combination with T3, the growth factors were applied at their optimal effective concentration of 1.4 nM for bFGF (22) and 50 nM for IGF I (23). Effects similar to those of T3 on α-sm actin accumulation and myofibrillar outgrowth have been found with bFGF in ARC cultured in the presence of unstripped FCS (22). Since T3 and bFGF use different intracellular signalling pathways and their effects may be additive, the combination of these two factors was tested at concentrations required for maximal stimulation of α-sm actin. In medium with stripped FCS, bFGF had no effect on α-sm actin mRNA expression, but it potentiated the stimulatory effect of T3 (Fig. 6, lanes *5* and *8*). Similarly, bFGF alone had no effect on the number of α-sm actin immunoreactive cells (Table III). In contrast to α-sm actin RNA expression, bFGF did not significantly increase the number of α-sm actin immunoreactive cells in T3-treated cultures. In order to determine whether bFGF stimulates α-sm actin expression only in the presence of T3 or if a "priming" of ARC with T3 is sufficient for bFGF action, bFGF and T3 were added sequentially in various combinations (Fig. 6). Again, the addition of bFGF alone during the whole culture period (d2–d13, lane *5*) or the second week of culture (d7–d13, lane *3*) had no effect on α-sm actin mRNA levels compared to controls (lane *1*). Culturing the cells with bFGF before T3-treatment (lane *7*) did not influence the stimulation observed with T3 alone (lanes *2* and *4*). In contrast, "priming" of ARC with T3 before the addition of bFGF resulted in α-sm actin mRNA levels above those observed with

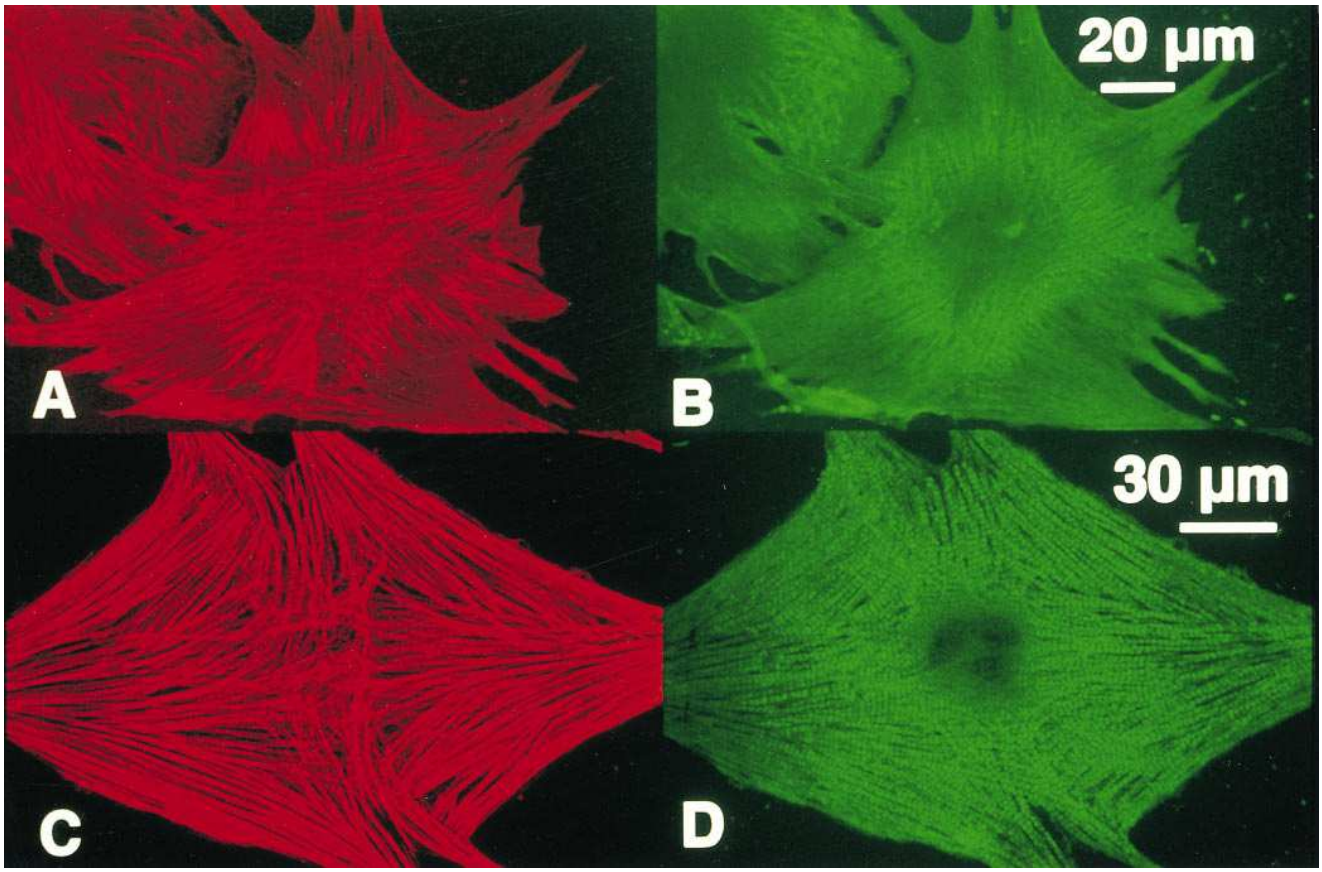

Figure 5. Confocal micrographs (layer at 0.3 μm from bottom) of adult rat cardiomyocytes after 10 d in culture double labeled with phalloidin-rhodamine for F-actin (*A* and *C*, *red*) and with a monoclonal antibody recognizing all myosin HC isoforms, pan-myosin (*B* and *D*, *green*). *A* and *B*, treated with 100 nM T3; *C* and *D*, control.

T3 alone (lane *6* compared with lanes *2* and *4*). This sequential treatment was even more effective than simultaneous treatment with T3 and bFGF during 2 wk (lane *8*). Hence, T3 seems to be permissive for the action of bFGF during 2 wk (lane *8*). Hence, T3 seems to be permissive for the action of bFGF on α-sm actin expression. Similar experiments were performed with IGF I. The results are summarized in Table IV. In one experiment, a low concentration of T3 (1 nM) was employed which corresponds approximately to the situation in unstripped FCS, while in the other two experiments with sequential application of the factors, T3 was present at a maximally active concentration (100 nM). In all three experiments IGF I had no effect on α-sm actin mRNA levels as compared to the untreated controls. However, IGF I abolished the stimulatory effect of 1 nM T3 when applied concomitantly. IGF I also reduced α-sm mRNA levels to almost those of controls when applied sequentially after 100 nM T3. Thus, T3 seems to be permissive for the action of IGF I as it is for bFGF.

Discussion

α-sm actin is a fetal actin isoform and is not expressed in adult cardiac tissue nor in freshly isolated ARC (34). Its reexpression together with β-MHC and atrial natriuretic factor in long-term cardiomyocyte cultures may represent partial reversion to a dedifferentiated, fetal phenotype (19, 24, 31). When ARC were cultured with stripped FCS, we could hardly detect any α-sm actin mRNA. However, α-sm actin mRNA was reexpressed after addition of T3. Hence, expression of α-sm actin in the presence of unstripped FCS appears to be due to its T3 content (around 0.6 nM in 20% FCS, table I). Induction of both α-sm actin mRNA and protein by T3 was dose-dependent and occurred in parallel suggesting regulation at the transcription level.

The gene for α-sm actin is also reexpressed in vivo in overload hypertrophy (35). Activation of genes encoding protein isoforms associated with embryonic stages may be a general event in the development of cardiac hypertrophy regardless of whether it is induced by volume or pressure overload or by hyperthyroidism. This would agree with our finding that T3 stimulates reexpression of α-sm actin.

In ARC cultures treated with 100 nM T3, 62% of all cells stained positively for α-sm actin. Staining was most intensive around the perinuclear region and tapered off into the periphery. It was almost superimposable with phalloidin staining which mainly stained stress fibre-like actin filaments leading from the central cell area to the cell periphery. These findings indicate that α-sm actin may be incorporated in the stress fibre-like actin structures. T3-treatment, like bFGF in unstripped FCS (22), induced a dramatic restriction of the myofibrils to the perinuclear area (Figs. 3 and 4). Double fluorescence images of α-sm actin and myofibrillar staining revealed that α-sm actin bundles start exactly where myofibrillar restriction ends (Fig. 4). It thus appears that the overexpression of α-sm actin

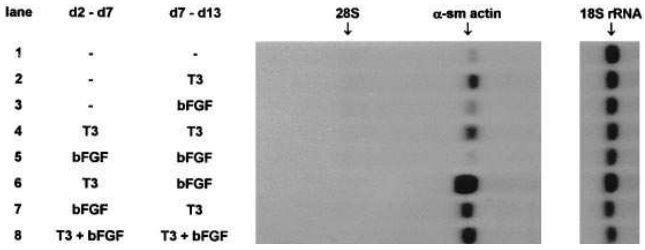

Figure 6. Permissive effect of T3 for bFGF action on α-sm actin mRNA expression. Lanes *1–8*: Northern blot analysis of 8 μm total RNA isolated from 13-d-old ARC after sequential addition of 100 nM T3 and 1.4 nM bFGF. One representative experiment out of three is shown. Variability of gel loading was checked by hybridization with an 18S rRNA specific probe.

observed with high concentrations of T3 coincides with restriction of myofibrillar growth. In addition to α-sm actin, T3 and bFGF may also induce the expression of other structural proteins which could be responsible for myofibrillar restriction. Such proteins may localize very specifically at the boundary between the myofibrils and the α-sm actin cables and inhibit outgrowth of myofibrils. Despite the restriction of the myofibrillar apparatus the T3-treated cells were beating vigorously. T3 may indirectly enhance beating rate by increasing the number of β-adrenergic receptors (36) and thus the responsiveness to catecholamines (37) probably present at low concentrations even in stripped FCS.

The morphological changes as well as the beating frequency, α-sm actin mRNA and protein accumulation are summarized and plotted semilogarithmically against the T3 concentration in the culture medium in Fig. 7. All parameters increased most steeply between 1 and 10 nM T3 and exhibited saturation at higher concentrations. The observed parameters were not visibly influenced whether the cells were isolated or whether they formed contacts with neighbouring cells.

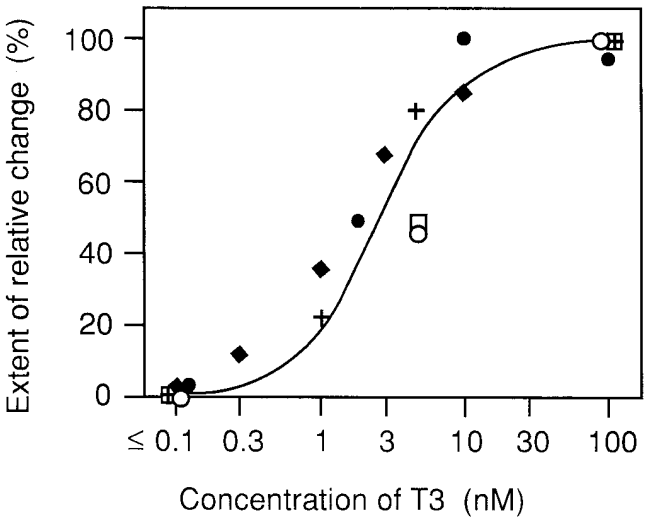

Figure 7. Semilogarithmic plot of changes of cell parameters in dependence on T3 in the medium of ARC after 12–14 d in culture. Extent of changes were normalized to 100% and plotted against increasing T3 concentrations (log scale). Cell beating frequency (●); α-sm actin mRNA (+); α-sm actin protein accumulation (◆); α-sm actin immunoreactive cells (○); cells with myofibrillar growth restriction (□).

Whereas α-sm actin expression and myofibrillar restriction are stimulated by bFGF in the presence of 10–20% unstripped FCS (22), bFGF had no effect on α-sm actin mRNA and protein expression in the presence of stripped FCS. However, treatment with bFGF and T3 together potentiated the stimulatory effect of T3. These results indicate that T3, either as a component of normal FCS or as a supplement to stripped FCS, is required for bFGF to enhance α-sm actin expression. Pretreatment with T3 before the addition of bFGF stimulated α-sm actin mRNA even more effectively than simultaneous addition of both factors. Hence, T3 appears to be permissive for the upregulation of α-sm actin by bFGF.

The results with IGF I demonstrate that also the downregulation of α-sm actin is dependent either on the presence of small concentrations of T3 or on the priming by T3 in sequential application (Table IV). Stimulation of myofibrillogenesis with concomitant downregulation of α-sm actin by IGF I reported by Donath et al. (23), may have been due to the presence of small amounts of T3 in unstripped FCS used in these studies.

It is intriguing that the described T3 and T3-primed bFGF actions in ARC are similar although the two factors use completely different intracellular signalling pathways. T3 binds to a nuclear receptor which acts directly on the DNA (38), whereas bFGF activates its receptor tyrosine kinase and triggers the Ras-Raf-MAP kinases pathway (39). The fact that T3 is a prerequisite for bFGF action implies some lateral communication between these pathways.

FGFs are produced by cardiomyocytes in vivo (40, 41) and seem to be associated with regenerative functions as well as with pathological events such as ischemia and hypertrophy (42). FGFs induce the expression of a number of fetal genes in neonatal and in adult cardiomyocytes (22, 43). This corresponds to the notion that FGFs are inhibitors of differentiation (44). In contrast, IGF I supports cell differentiation (44, 45), which is consistent with the recent finding that it enhances myofibrillar growth and down-regulates α-sm actin in ARC (22, 23).

In conclusion, T3 seems to be permissive for the opposite effects of bFGF and of IGF I on ARC in culture. This suggests that T3 may affect a step common in the signal pathway of

Table IV. Effects of IGF I on α-smooth Muscle Actin mRNA in Dependence of T3 after Simultaneous and after Sequential Application to ARC 13 d in Culture

Period of treatment		Experiments		
2–7 d	7–13 d	Nr. 1	Nr. 2	Nr. 3
—	—	32		
1 nM T3	1 nM T3	100		
50 nM IGF	50 nM IGF	37		
1 nM T3	1 nM T3			
+50 nM IGF	+50 nM IGF	29		
—	—		3.4	59
100 nM T3	—		100	100
—	50 nM IGF I		10	69
100 nM T3	50 nM IGF I		41	31

OD-values of the 2.1-kb α-sm actin mRNA bands were measured by scanning densitometry. RNA data were normalized against 18S rRNA values. Treatment with T3 alone is defined as 100%, relative values are given in percents.

both growth factors. The correlation of α-sm actin expression and myofibrillar restriction as observed under different experimental conditions (treatment with IGF I, bFGF, and T3) suggests that accumulation of α-sm actin in stress fiber-like structures blocks myofibrillar outgrowth.

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