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Cell Biology

Cardiac fibroblasts are predisposed to convert into myocyte phenotype: Specific effect of transforming growth factor β

(heart fibroblast/actin/differentiation)

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ABSTRACT **Cardiac fibroblasts are mainly responsible for the synthesis of major extracellular matrix proteins in the heart, including fibrillar collagen types I and III and fibronectin. In this report we show that these cells, when stimulated by transforming growth factor β_1 (TGF-β_1), acquire certain myocyte-specific properties. Cultured cardiac fibroblasts from adult rabbit heart were treated with TGF-β_1 (10–15 ng/ml) for different periods of time. Northern hybridization analysis of total RNA showed that cells treated with TGF-β_1 for 24 hr expressed mRNA corresponding to sarcomeric actin mRNA. Immunofluorescence staining and light microscopy showed that cultured cardiac fibroblasts treated with TGF-β_1 became stained with a monoclonal antibody to muscle-specific actin. After treatment of quiescent cells with TGF-β_1, cell proliferation (as measured by [^{3}H]thymidine incorporation) was moderately increased (1.5-fold, $P < 0.001$). NIH 3T3 cells and human skin fibroblasts, treated with TGF-β_1, did not express sarcomeric actin mRNA. Treatment of cardiac fibroblasts with the mitogenic agent phorbol 12-myristate 13-acetate or with norepinephrine, angiotensin II, or interleukin 1β did not induce myocyte-specific actin mRNA. Cultured cardiac fibroblasts at the subconfluent stage, when exposed to TGF-β_1 in the presence of 10% fetal bovine serum, gave rise to a second generation of slowly growing cells that expressed muscle-specific actin filaments. Our findings demonstrate that cardiac fibroblasts can be made to differentiate into cells that display many characteristics of cardiac myocytes. TGF-β_1 seems to be a specific inducer of such conversion.**

The myocardial cell population consists largely of cardiac myocytes that occupy ≈90% of the myocardial mass. Ninety to 95% of the nonmyocyte fraction of cardiac cells consists of cardiac fibroblasts (1, 2).

Little is known about cardiac myogenesis. No stem cell has been identified and no cell line has been found that will convert into cardiac myocytes upon specific external stimuli.

A highly significant finding in the last few years has been the discovery of the myoD family of genes that is able to promote conversion of fibroblasts into skeletal muscle cells (3). It would be highly desirable to develop a similar system for cardiac myocytes.

(TGF-β_1) Transforming growth-factor β_1 has been shown to act as a mitogen and growth factor for various cultured cells. This growth factor is present in various tissues (4, 5). We (6) and others (7) have previously reported the presence and distribution of TGF-β_1 in the rat myocardium. The purpose of the present study was to examine the effects of TGF-β_1 on cardiac fibroblasts with respect to growth and differentiation. In this report, we present evidence indicating that cardiac fibroblasts obtained from hearts of adult rat, when exposed to TGF-β_1, can differentiate into cells that display some of the phenotypic features of cardiac myocytes.

METHODS

Cell Culture and Treatments. Adult male (New Zealand White) rabbits were anesthetized; hearts were excised, minced, and washed in phosphate-buffered saline (PBS). The tissue was treated at 35°C with a mixture of 0.1% trypsin and 100 units of collagenase per ml (type IV, Sigma) for 10 min. Isolated cells were pelleted at the end of several 10-min digestion periods, plated on 100-mm culture dishes in Dulbecco's modified Eagle medium (DMEM) containing 10–15% fetal bovine serum (FBS), and incubated for 2 hr at 37°C in an incubator with 90% O_2/10% CO_2 with a humidifier. At the end of that period, the unattached cells were discarded and attached cells were grown in DMEM with 10% FBS. The fibroblastic nature of confluent cells as well as the purity of the cultured cell population was determined by immunofluorescence staining of the cell layer with anti-human factor VIII antibody (Behring Diagnostics) for endothelial cells, anti-desmin (Calbiochem) for muscle cells, and anti-vimentin (2) for fibroblasts. In a confluent cell preparation only 1–2% of cells stained positively with anti-factor VIII and anti-desmin. All cells were stained positively with anti-vimentin antibody. For all our studies cells from passages 4–6 were used. For each treatment, cells were grown to confluency in medium containing 10% FBS. They were then deprived of serum for 24 hr. The growth factor and other agents were then added as follows: TGF-β_1 (R & D Products, Rochester, MN), 10–15 ng/ml; phorbol 12-myristate 13-acetate (PMA) (Sigma), 200 ng/ml; angiotensin II (AII) (Sigma), 1 μM; norepinephrine (NE) (Sigma), 1 μM; interleukin 1β (IL-1β) (R & D Products), 1 ng/ml.

To study the possible additive effects on cell growth of TGF-β_1 and FBS, subconfluent cardiac fibroblasts were treated with TGF-β_1 in the presence of 10–15% FBS for 24 hr. Cells were then washed with fresh medium and incubation was continued in serum-supplemented medium for 24 hr. Cells were then passaged at low density and their growth and morphology were examined over a 2-week period of time. Parallel experiments were performed without addition of TGF-β_1.

Endothelial (8) and smooth muscle cells (9) had been isolated from bovine aorta and cultured. They were used in passages 12–28 in the case of endothelial cells and in passage 2 in the case of smooth muscle cells. Both cell culture preparations were generously supplied by Peter Davies (University of Chicago). The RNA extracted from these cells was used as a negative control and for detection of a sarcomeric actin mRNA. To examine the cell type specificity, parallel

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Abbreviations: TGF, transforming growth factor; PMA, phorbol 12-myristate 13-acetate; FBS, fetal bovine serum; IL, interleukin; NE, norepinephrine; AII, angiotension II.

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experiments with TGF-β_1 were performed using human skin fibroblasts (American Type Culture Collection CRL 1106) and mouse NIH 3T3 cells (Vikas P. Sukhatme, University of Chicago).

RNA Extraction and Northern Analysis. RNA was extracted from confluent cell layers in 100-mm culture dishes by the method of Chomczynski and Sacchi (10). In the case of ventricular tissue, RNA was extracted according to the method of Chirgwin *et al.* (11) as described (1).

Total RNA was then analyzed by gel electrophoresis on a 1% agarose gel containing formaldehyde. Recombinant plasmids and oligonucleotides used as probes were as follows: a full-length cDNA probe to chicken β-actin (12) and 20-mer oligonucleotides, TTGTGGGATAGCAACAGCGA and GGTCTCAGGGCTTCACAGGC, complementary to a portion of the 3′ untranslated regions of rat α- and β-myosin heavy chain mRNAs, respectively (13). The cDNA probe was labeled with [^{32}P]dCTP by the method of Feinberg and Vogelstein (14), using the multiprime DNA labeling system (Amersham). Blot transfer and hybridization procedures for the cDNA probe have been described (1). Oligonucleotides were labeled with [^{32}P]ATP using a 5′ end-labeling biosystem (IBI). Prehybridization, hybridization, and washing procedures were according to methods previously described (13). After hybridization, membranes were washed and exposed to Kodak XAR-5 film at −70°C.

Immunofluorescent Staining. Cardiac fibroblasts were cultured until confluent in DMEM and 10% FBS on Lab-Tek Permanox chamber slides (Nunc) or on 60-mm culture dishes. For cell characterization studies, confluent cells in medium containing serum were used. For TGF-β_1 treatment, they were deprived of serum for 24 hr. TGF-β_1 was then added to the culture medium. At the end of treatment (24–72 hr), cell layers were washed twice with PBS and permeabilized in absolute methanol for 7 min. Staining of cell layers was performed as described (15). Briefly, cells were incubated with a 1:50 dilution of monoclonal antibodies to muscle-specific actin or smooth muscle-specific actin (Enzo Diagnostics), polyclonal rabbit anti-human factor VIII (Behring Biochemicals), rabbit anti-desmin (Calbiochem), or a polyclonal rabbit anti-vimentin antibody (1) for 1 hr and washed with PBS. For a negative control, IgGs prepared from mouse or rabbit preimmune serums were used. Fluorescein isothiocyanate-conjugated goat anti-rabbit and anti-mouse IgGs were used as second antibodies. Visualization was performed by the use of an Olympus model BH_2 epi-fluorescent microscope.

[^{3}H]Thymidine Incorporation. Cardiac fibroblasts were grown until confluent in DMEM and 10% FBS on 60-mm culture dishes. They were then serum-deprived for 24 hr. TGF-β_1 was added to the culture medium. Treatment lasted for 22 hr. [^{3}H]Thymidine (5 μCi/ml; 1 Ci = 37 GBq; specific activity, 86 Ci/mmol; Amersham) was added to the medium. After 2 hr, cells were washed three times with PBS and lysed in 0.1% sodium dodecyl sulfate/0.1 M NaOH. DNA was precipitated by 5% trichloroacetic acid (TCA). The precipitate was filtered and filters were washed with 10% TCA and 95% ethanol. The radioactivity was determined by liquid scintillation spectroscopy (16). DNA was quantified by the use of a chromogenic reagent (0.1 ml of acetaldehyde/1.5% diphenylamine in glacial acetic acid and concentrated sulfuric acid) (17).

RESULTS

Induction of Muscle-Specific mRNAs in Cardiac Fibroblasts. The first evidence of potential myogenesis by TGF-β_1 was the observation that quiescent confluent cardiac fibroblasts, upon treatment with TGF-β_1, acquired morphological features such as an elongated shape in contrast to the normal star-shaped morphology characteristic of quiescent cardiac fibroblasts. Also, they had become assembled into organized bundles along their long axes (see Fig. 4). We therefore asked whether these fibroblasts were possibly converting to a muscle phenotype and studied potential mechanisms that could lead to such muscle-like morphological changes. First, we searched for the expression of muscle-specific mRNAs in the TGF-β_1-treated cells. Total RNA extracted from these cells was subjected to Northern hybridization analyses with a cDNA probe to chicken β-actin and with oligonucleotides complementary to α- and β-myosin heavy chains. The use of a β-actin cDNA probe was based on the fact that, due to the high degree of sequence conservation among actin isoforms, the cDNA probe to β-actin cross-hybridizes to sarcomeric actin mRNAs if these are present (18). Therefore the use of this probe allows the detection of sarcomeric actin and provides extra assurance that myocytes are not present in the untreated cardiac fibroblast population in culture. The results of hybridization analysis from five individual experiments revealed that untreated control cells expressed only one actin isoform mRNA. However, after 24 hr TGF-β_1-treated cells exhibited definite induction of an mRNA band that cross-hybridized with a β-actin cDNA probe. That band corresponded in size to the sarcomeric actin mRNA and exhibited a pattern identical to that observed in RNA extracted from cardiac ventricular tissue (Fig. 1). Induction of the new actin mRNA was not observed at 3 hr of treatment with the same dose of TGF-β_1 (Fig. 1). The intensity of the induced mRNA band was increased after longer periods (72 hr) of TGF-β_1 treatment. Hybridization analysis of RNA extracted from confluent cells in serum-supplemented medium showed barely visible bands corresponding to the induced actin mRNA band (data not shown). Cardiac fibroblasts that exhibited the response to TGF-β_1 had been deprived of serum for 24 hr.

To determine further the specificity and the nature of the induced actin mRNA, and to rule out the possibility of conversion of the fibroblasts into smooth muscle or endothelial cells, total RNAs extracted from endothelial and smooth muscle cells were analyzed by Northern hybridization with a cDNA probe to β-actin. Autoradiography revealed that in those cells the cDNA probe detected only one mRNA band with a size corresponding to the cytoskeletal actin mRNA, which gave a pattern similar to that of nonmuscle actin mRNA observed with RNA extracted from ventricular tissue (Fig. 2).

When we searched for the expression of α- and β-myosin heavy chain mRNAs, a faint band corresponding to the mRNA for β-myosin heavy chain was observed in Northern hybridization analysis of RNA from TGF-β_1 treated cells. However, the pertinent band did not increase in density after longer periods (72 hr) of treatment with TGF-β_1 (data not

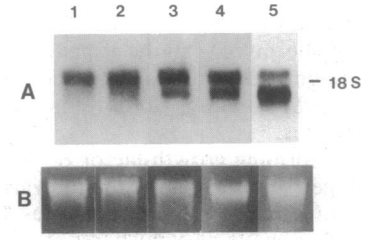

FIG. 1. Autoradiograms of Northern hybridization analysis of total RNA. Total RNA was extracted from confluent quiescent rabbit cardiac fibroblasts and from rat ventricular tissue. Ten micrograms of RNA was then subjected to size fractionation on a 1% agarose gel. (*A*) Hybridization of mRNA to a ^{32}P-labeled cDNA probe to chicken β-actin is shown for untreated cells (lane 1), 3-hr TGF-β_1 treatment (lane 2), 24-hr TGF-β_1 treatment (lane 3), 72-hr TGF-β_1 treatment (lane 4), and ventricular tissue (lane 5). (*B*) Corresponding 28S rRNA bands that are visualized by ethidium bromide staining. Similar autoradiograms were obtained in three individual experiments.

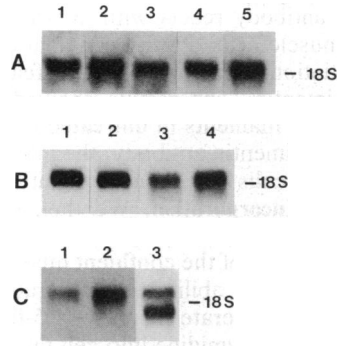

FIG. 2. Northern hybridization analysis of total RNA. Total RNA was extracted from confluent quiescent cardiac fibroblasts, NIH 3T3 cells, bovine smooth muscle and endothelial cells, and rat ventricular tissue. Ten micrograms of RNA was analyzed and hybridized to a ^{32}P-labeled cDNA probe to chicken β-actin. (*A*) mRNA hybridization of untreated cells (lane 1), NE-treated cells (lane 2), AII-treated cells (lane 3), PMA-treated cells (lane 4), and IL-1β-treated cells (lane 5) with β-actin cDNA probe. (*B*) Cytoskeletal actin mRNA detected by the use of β-actin cDNA probe in TGF-β_1-treated mouse NIH 3T3 cells (lane 2), human skin fibroblasts (lane 4), and corresponding untreated cells (lanes 1 and 3). (*C*) Presence of a cytoskeletal actin mRNA band in RNA extracted from endothelial cells (lane 1) and smooth muscle cells (lane 2). Sarcomeric and cytoskeletal actin mRNA bands in RNA extracted from ventricular tissue are shown in lane 3.

shown). No hybridization signals were detected for α-myosin heavy chain mRNA.

Specificity of the Effect of TGF-β_1 on Cardiac Fibroblasts. The specificity of the observed TGF-β_1 effect was examined by treatment of cardiac fibroblasts with PMA, an agent with known mitogenic and growth-enhancing properties in various cell types. Treatment of quiescent confluent cells with PMA for 24 hr did not induce the myocyte-specific mRNA (Fig. 2). We also asked if other cytokines such as IL-1β could have the same effect as TGF-β_1 on cardiac fibroblasts. Treatment of cardiac fibroblasts for 24 hr with IL-1β did not result in the induction of sarcomeric actin mRNA (Fig. 2). In the living myocardium, cardiac cells are usually exposed, in addition to TGF-β_1, to an array of hormones and neurotransmitters, including AII and NE. These substances either are produced in the myocardium or gain excess to the tissue by way of circulating blood. We asked if these agents could have effects similar to those of TGF-β_1 with regard to the induction of sarcomeric actin. Quiescent confluent cardiac fibroblasts were therefore treated with AII or NE for 24 hr. Neither agent induced the sarcomeric actin mRNA (Fig. 2).

As a control for cell type specificity, mouse NIH 3T3 cells and human skin fibroblasts were used. Treatment of quiescent confluent NIH 3T3 cells or human skin fibroblasts with TGF-β_1 for 24 hr did not induce sarcomeric actin mRNA (Fig. 2).

Morphology of Cardiac Fibroblasts Following TGF-β_1 Treatment. Quiescent confluent rabbit cardiac fibroblasts were treated for 24 hr with TGF-β_1. At the end of that period, the morphology of the cells as examined by phase-contrast light microscopy was completely different from that of corresponding untreated cells (Fig. 3). Unlike untreated fibroblasts, which usually have a star shape and spread-out

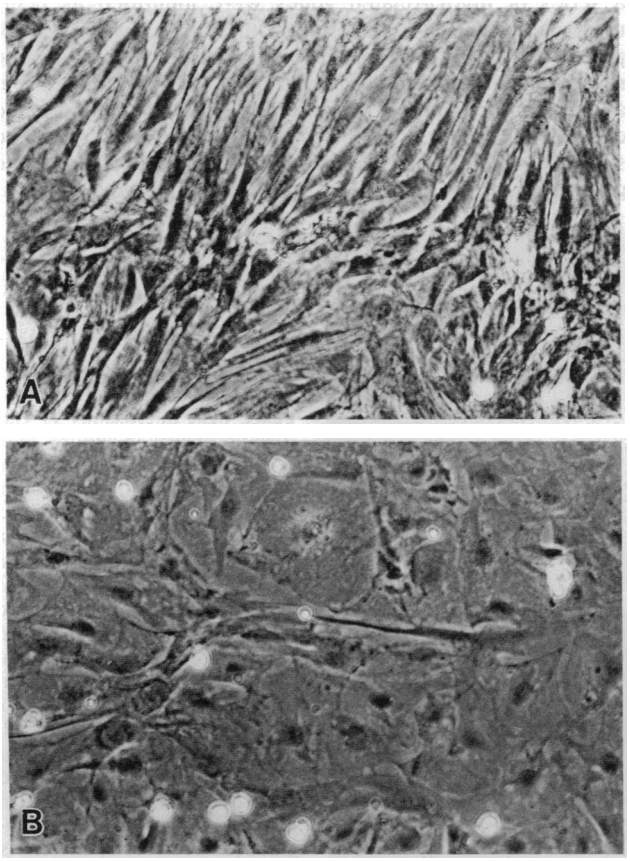

FIG. 3. Phase-contrast photomicrographs of rabbit cardiac fibroblasts. Confluent quiescent cardiac fibroblasts were treated with TGF-β_1 for 24 hr (*A*) or received no treatment (*B*). Photographs were taken of unfixed cells in culture medium. (×172.)

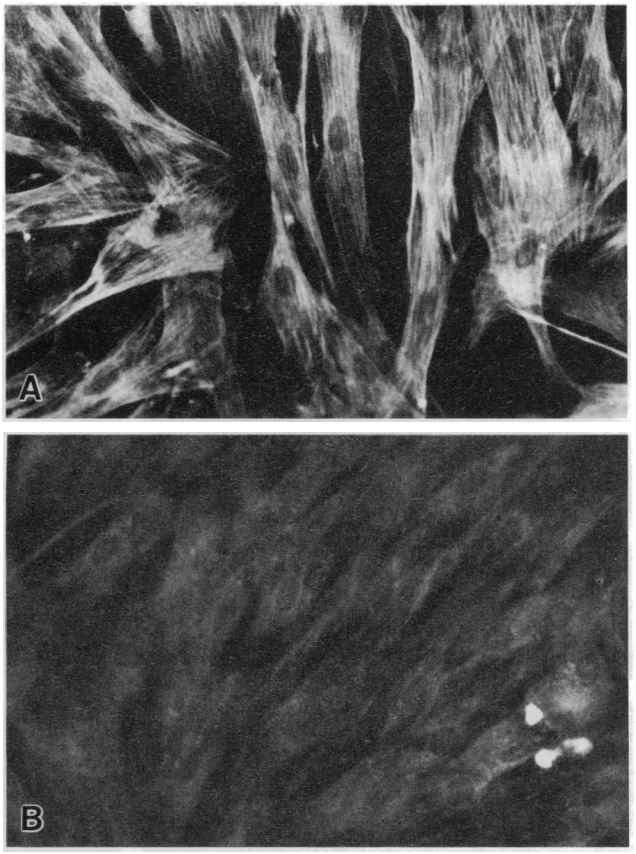

FIG. 4. Immunofluorescent photomicrographs of rabbit cardiac fibroblasts. Confluent quiescent cardiac fibroblasts were washed with PBS two times and fixed and permeabilized with absolute methanol for 7 min. Cells were then stained with monoclonal antibody to muscle-specific actin. (*A*) Actin filaments in cells after a 24-hr treatment with TGF-β_1. (*B*) Untreated cells showing no significant immunoreactivity with anti-muscle-specific actin antibody. (×172.)

morphology, TGF-β_1-treated cells acquired an elongated shape. A very distinctive feature of TGF-β_1-treated cells in culture was their assembly into organized bundles of cells along their long axes. A similar pattern is indeed observed in the organization of cardiac myocytes seen in cardiac ventricular tissue. Cardiac fibroblasts in presence of FBS (10–15%) also showed an elongated morphology different from that of quiescent cells (data not shown).

Visualization of Muscle-Specific Actin Filaments. Immunofluorescent light microscopy clearly revealed the actin filaments in cells treated with TGF-β_1 for 24 hr or more. These filaments were absent in untreated quiescent fibroblasts (Fig. 4). Serum-treated cells also expressed positive staining with muscle-specific actin antibody (data not shown). To establish the nature of the actin filaments, TGF-β_1-treated and untreated cells were stained with monoclonal antibody to smooth muscle actin. Less than 5% of cells in either group showed positive staining.

To examine the additive effects of TGF-β_1 with respect to serum on normal cell growth in a population of cells, TGF-β_1 was added to subconfluent cultures in which the medium already contained serum. Interestingly, subconfluent cells, when exposed to TGF-β_1 for 24 hr, in subsequent passage gave rise to a second generation of cells that had a very slow rate of proliferation and were considerably enlarged. Most importantly, when stained with anti-muscle-specific actin antibody, these cells expressed highly dense and visible actin filaments. Several of the treated cells appeared to have double nuclei, or their assembly with neighboring cells had created such an appearance (Fig. 5). We then studied whether these changes were due to the low density of the cells in the culture dishes since diminished contact could possibly induce morphological conversion. Therefore, cells plated in low density were treated with FBS only and their behavior was observed. Such cells showed no significant change of their usual morphology and continued to grow until confluent.

To study the extent of phenotypic conversion of the cardiac fibroblasts after treatment with TGF-β_1, cells were stained with anti-vimentin antibody. Vimentin is an intermediate-sized filament expressed by most cells in culture. Anti-vimentin antibody reacts with intermediate-sized filaments in nonmuscle cells of mesenchymal origin (19). Terminal differentiation of myoblasts usually features decreased amounts of vimentin. The results showed that, although intermediate-sized filaments in untreated cells were stained clearly with anti-vimentin antibody, the intensity of staining in TGF-β_1-treated cells was reduced significantly (Fig. 6).

[^{3}H]Thymidine Incorporation. We then studied whether converted cells were capable of proliferation. It was shown that TGF-β_1 treatment of the confluent quiescent cells for 24 hr did not abolish their ability to proliferate. Indeed, such treatment led to a moderate increase (1.5-fold, $P < 0.001$) incorporation of [^{3}H]thymidine into cell nuclei compared to incorporation in untreated cells (Fig. 7).

DISCUSSION

The results of our present study led to two major conclusions: First, rabbit cardiac fibroblasts are predisposed to differentiate into cardiac muscle cells. Second, TGF-β_1, endogenous in the myocardium, may prove to be the specific inducer of such phenotypic conversion of cardiac fibroblasts in culture. Several lines of evidence obtained in this study support these conclusions: (*i*) appearance of a sarcomeric actin mRNA in cardiac fibroblasts treated with TGF-β_1; (*ii*) appearance of muscle-specific actin filaments in such treated cells, as shown by immunofluorescent microscopy; (*iii*) induction of morphological features characteristic to myocytes in such treated cells; (*iv*) loss of intermediate filament vimentin; and (*v*) continued expression of muscle-specific features in second generation of cells stemming from TGF-β_1-treated cells.

Previously, 5-azacytidine has been shown to induce conversion of C2H10T½ fibroblasts into skeletal myocytes, and a series of myoD-related genes were identified as being responsible for the conversion. Our findings with TGF-β_1 present the possibility that the conversion of fibroblasts into myocytes can be studied *in vivo* or at the tissue level. Yet another major aspect of the present findings is the continued expression of muscle-specific morphological properties and slow growth and expression of actin filaments in the second generation of cells stemming from TGF-β_1-treated cells. Thus

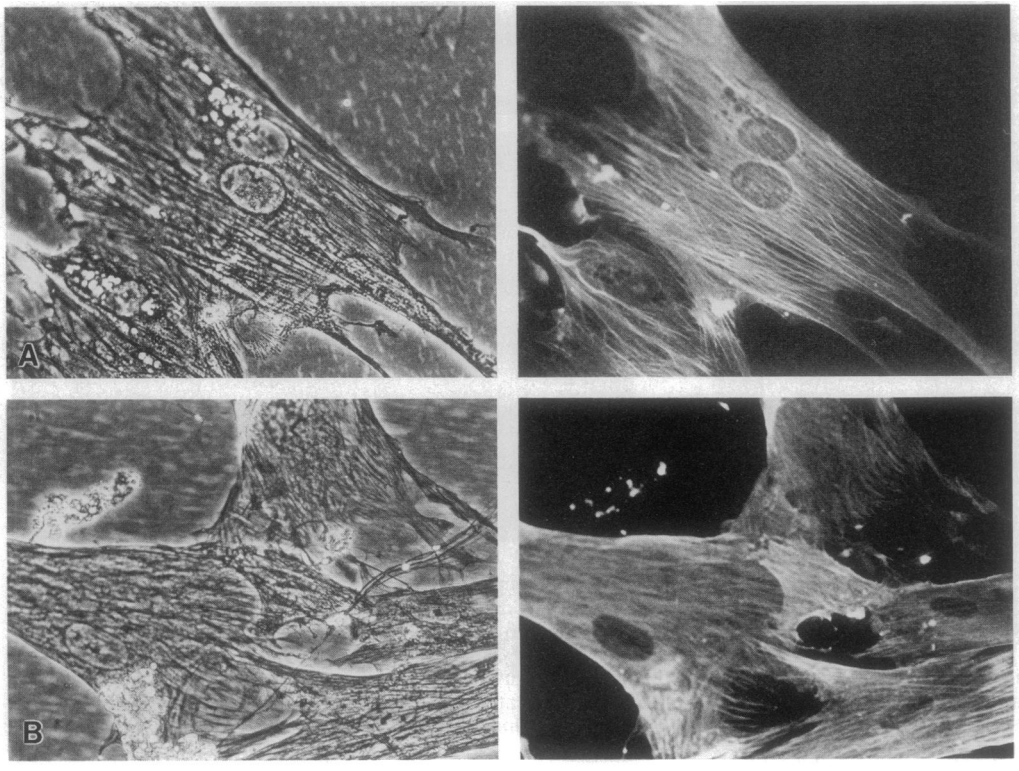

FIG. 5. Immunofluorescence light microscopy of cardiac fibroblasts in culture. Subconfluent cardiac fibroblasts in serum-supplemented medium were treated with TGF-β_1 (15 ng/ml) for 24 hr. Cells were then washed with DMEM and grown for another 24 hr. These cells were then passaged and seeded with low density in a 60-mm culture dish in DMEM containing 15% FBS; growth was continued over a period of 1–2 weeks. At the end of this period, cells were washed, fixed and permeabilized, and then stained with monoclonal antibody to muscle-specific actin. (*A*) Longitudinal assembly of the cells is clearly visible in immunofluorescent (right) and phase-contrast (left) photomicrographs. (*B*) Assembly of the cells in a different orientation. (×180.)

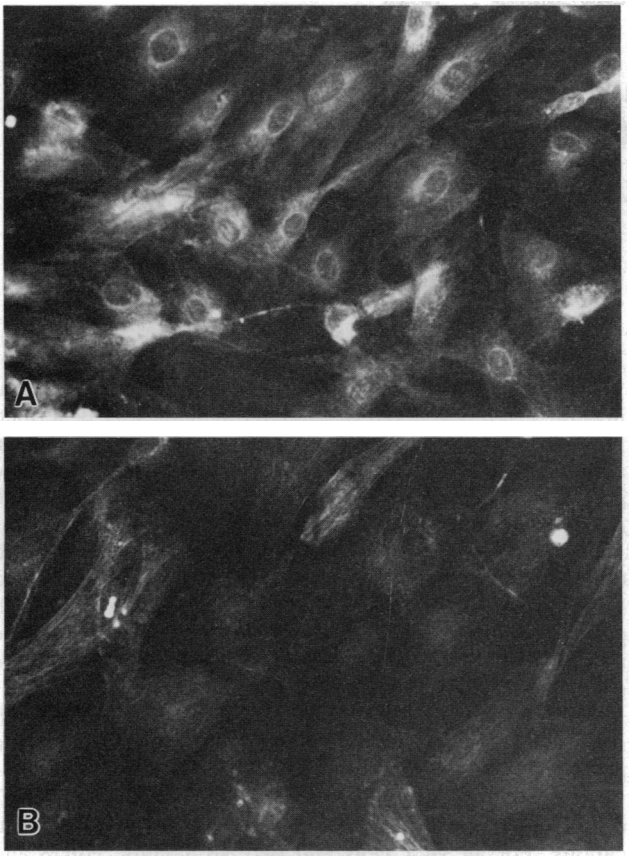

FIG. 6. Immunofluorescence light microscopy of rabbit cardiac fibroblasts. Confluent quiescent cardiac fibroblasts were treated with TGF-β_1 (*A*) or received no treatment (*B*). After a 24-hr treatment, cells were washed, fixed and permeabilized, and then stained with a 1:50 dilution of rabbit anti-vimentin antibody. (×172.)

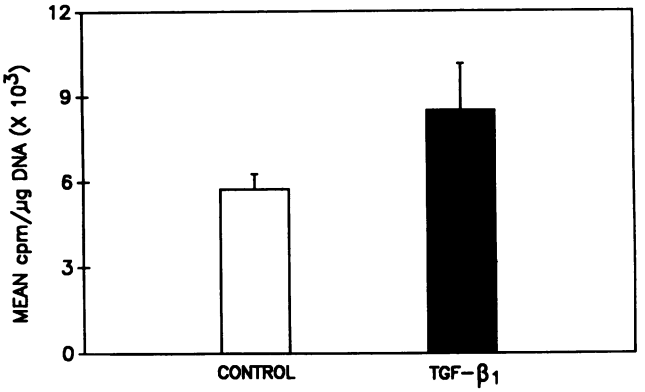

FIG. 7. [^{3}H]Thymidine incorporation into cell nuclei. Confluent quiescent cardiac fibroblasts were treated with 10–15 ng of TGF-β_1 per ml for 22 hr. [*methyl*-^{3}H]Thymidine (5 μCi/ml) was added to the medium. Cells were harvested after 2 hr and [^{3}H]thymidine incorporation was measured per μg of DNA in the cell lysate. Statistical analysis of data was performed using Student's *t* test for group data. Data are expressed as means ± SD (n = 3). $P < 0.001$.

it would appear that phenotypic conversion of cardiac fibroblasts due to TGF-β_1 treatment persists in succeeding generations. NIH 3T3 cells and human skin fibroblasts did not respond to TGF-β_1 by expressing sarcomeric actin mRNA, showing that not all fibroblasts exhibit a predisposition for conversion into differentiated cells when treated with TGF-β_1. However, that matter requires more detailed study. To be fully substantiated, the concept of conversion of cardiac fibroblasts into cardiac myocytes requires future documentation of definite expression of myosin heavy chains and beating capacity of these cells. Our studies with smooth muscle cell-specific actin antibody showed no significant expression of this actin isotype in treated cells. Furthermore, the sarcomeric actin mRNA band seen in TGF-β_1-treated cells was not detected in RNA extracted from smooth muscle cells. In the course of our studies TGF-β_1 was shown to stimulate biosynthesis of fibrillar collagens in cardiac fibroblasts (unpublished data). Since fibrillar collagen biosynthesis is characteristic of cardiac fibroblasts in culture, it seems that TGF-β_1, on the one hand, induces a phenotype characteristic of cardiac myocytes and, on the other hand, potentiates certain residual properties of cardiac fibroblasts such as the synthesis of specific collagens. The physiological significance of such a dual effect of TGF-β_1 remains to be clarified. Experiments involving [^{3}H]thymidine incorporation demonstrated the capacity of converted cells to proliferate. Although these results suggest that the treated cells do not terminally differentiate into myocytes, they also allow the tantalizing possibility that treatment with TGF-β_1 leads to induction of cells with a cardiac myocyte phenotype that also are capable of growth and proliferation.

Our findings suggest that combinations of TGF-β_1 and other factors normally present in cardiac tissue may be exploited to cause a more complete phenotypic conversion of cardiac fibroblasts into cardiac myocytes. Furthermore, in view of the fact that the conversion reported here occurs in cardiac fibroblasts from adult animals, the physiological and clinical impact of these findings could be highly significant since they suggest the possibility that new populations of cardiac myocytes could be generated from fibroblasts in adult heart as a partial compensation for the lack of regenerative capacity in differentiated adult cardiac myocytes.

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