Role of Transforming Growth Factor-β in Maintenance of Function of Cultured Neonatal Cardiac Myocytes

Autocrine Action and Reversal of Damaging Effects of Interleukin-1

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Abstract

The three isoforms of transforming growth factor-β (TGF-β) have previously been implicated in embryonic development of the heart as well as in repair of myocardial damage after ischemia/reperfusion injury. TGF-β1 has also been localized intracellularly to both mitochondria and contractile filaments of cardiac myocytes, although its role in these structures has not been defined. We now report that exogenous TGF-β stabilizes the beating rate of neonatal rat cardiac myocytes cultured on fibroblast matrix, and sustains their spontaneous rhythmic beating in serum-free medium. Moreover, using blocking antibodies to TGF-β, we show that endogenous TGF-β secreted by these myocytes acts in an autocrine fashion to maintain their beating rate. In contrast, IL-1β, an inflammatory mediator secreted by immune cells during myocardial injury, inhibits the beating of cardiac myocytes, and TGF-β can overcome this inhibition. The antagonistic effects of TGF-β and IL-1 were not observed when the myocytes were cultured on gelatin, as compared to native fibroblast matrix. The data indicate that TGF-β is an important regulator of contractile function of the heart and have significant implications for understanding cardiac physiology in health and disease. (*J. Clin. Invest.* 1992. 90:2056–2062). Key words: transforming growth factor-β • interleukin-1 • heart • myocardium • extracellular matrix

Introduction

Little is known regarding the roles of peptide growth factors (cytokines) in regulating cardiac function, although it is known that the myocardium produces several growth factors and that their expression is highly regulated during both embryonic development and disease in the adult (1). One of these cytokines, transforming growth factor-β (TGF-β),[1] is expressed at high levels in the heart during both embryonic and adult life (2, 3). In embryonic development of the heart, TGF-β has been implicated both in morphogenesis of the cushion tissue which will form the heart valves and in the epithelial-to-mesenchymal transformation of valve progenitor cells (4–7). The three isoforms of TGF-β, TGF-β's 1, 2, and 3, whose biological activities in vitro are most often indistinguishable (8), are differentially expressed during early development of the heart. However, at later stages of embryogenesis and in the adult, all three isoforms of TGF-β are expressed in the myocardium (K. Flanders, personal communication).

TGF-β plays a central role in the repair of many tissues after injury (8). Recently, attention has turned to its effects in repair or modulation of cardiac tissue damage resulting from myocardial ischemia or infarction. Studies on the immunohistochemical staining patterns of TGF-β1 in the heart after experimental myocardial infarction have demonstrated that staining for TGF-β1 is quickly lost in ischemic areas (2). However, certain cells around the margins of the infarct begin staining intensely for TGF-β1 ~ 24–48 h after infarction, leading to the suggestion that TGF-β might accelerate repair and restore function to these myocytes. Recent electron immunohistochemical and parallel cell fractionation studies have shown specific subcellular localization of TGF-β1 in mitochondria and contractile filaments of cardiac myocytes (9). These findings suggest that TGF-β has an intrinsic, physiological role in myocytes, which is presently unknown.

Based on these observations, we postulated that TGF-β might have an intrinsic role in regulating either cardiac myocyte contractility or energy metabolism (9). Moreover, because of our interest in defining the roles of TGF-β in acute and chronic cardiac injury, we have examined whether exogenous TGF-β might have direct effects on myocyte function. Since mediators of acute and chronic cardiac damage, such as IL-1, have been shown to have suppressive effects on myocyte function (10), we also investigated whether TGF-β might antagonize these effects of IL-1, analogous to its ability to oppose many of the actions of IL-1 on other cell types (11–14). We have used cultures of neonatal rat cardiac myocytes which beat spontaneously as a measure of myocyte function, and in this system, show that TGF-β plays an intrinsic role in stabilizing their beating rate in serum-free culture and that it is protective against the suppressive effects of IL-1 on the contractility of myocytes.

Methods

Reagents. Porcine TGF-β1 and 2 were obtained from R & D Systems, Inc. (Minneapolis, MN). Recombinant chicken TGF-β3 was expressed from a metallothionein promoter in NIH 3T3 cells and purified to homogeneity as described (15). Unless otherwise stated, TGF-β1 was used for all experiments. Recombinant human IL-1β was obtained from Michael Palladino, Genentech, Inc. (South San Francisco, CA); murine epidermal growth factor (EGF) was purified from salivary glands as described (16); recombinant bovine basic fibroblast growth factor (bFGF) was obtained from Amgen Biologicals (Thousand Oaks, CA), and recombinant human platelet-derived growth factor (PDGF-BB) was obtained from Mark Murray, Zymogenetics, Inc. (Seattle, WA). All growth factors were stored in concentrated (10 to 40

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1. *Abbreviations used in this paper:* bFGF, basic fibroblast growth factor; EGF, epidermal growth factor; PDGF, platelet-derived growth factor; TGF-β, transforming growth factor-β.

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μg/ml) solutions at either −70° (IL-1β) or −20°C; at the time of the experiment the solutions were diluted to 50× final concentration in PBS containing 1 mg/ml bovine serum albumin, and added to the cultures.

Specific turkey and rabbit polyclonal antibodies against TGF-β1 and 2, respectively, have been previously described (17). A monoclonal antibody that blocks the activity of all 3 TGF-β isoforms was obtained from Genzyme Corp. (Cambridge, MA) and purified mouse ascites fluid IgG1 used as a control was obtained from Chemicon International Inc. (Temecula, CA). Assay kits were used to determine ATP (luciferase assay; LKB Instruments, Inc., Gaithersburg, MD) and cAMP (binding assay; Amersham Corp., Arlington Heights, IL).

Preparation of neonatal cardiac myocytes. Myocytes were prepared from 2-d-old rat pups, using modifications of previously described methods (18). Briefly, the dissected left ventricles of 30–60 hearts were minced in PBS (without Ca^{++} and Mg^{++}) containing 1% glucose, and digested for 10-min periods in a 50-ml spinner flask (Bellco Biotechnology, Vineland, NJ) in 10 ml of a solution consisting of 40 ml 0.25% trypsin (Gibco Laboratories, Grand Island, NY), 60 ml Hanks' salts without Ca^{++} and Mg^{++} (Gibco Laboratories) to which $MgCl_2$ was added back to 0.81 mM, 3,500 U collagenase (type 2; Worthington Biochemical Corp., Freehold, NJ) and 10 mg deoxyribonuclease I (Worthington Biochemical Corp.). Digestion was stopped by adding 5 ml of PBS (without Ca^{++} and Mg^{++}) containing 2% calf serum, 0.01% deoxyribonuclease, 1% glucose. Cycles of digestion were continued until no more tissue remained.

After a differential plating to remove fibroblasts, myocytes were further purified from nonmyocytes on a gradient system consisting of Percoll (Sigma Chemical Co., St. Louis, MO) in Minimal Essential Medium (Gibco Laboratories) adjusted to final densities 1.082, 1.061, and 1.050 g/ml (19, 20). The purified myocytes banding between the 1.082 and 1.061 g/ml layers were cultured in Dulbecco's Modified Eagle's Medium/F12 (DMEM/F12) containing 10–15 mM Hepes buffer (Gibco Laboratories) and supplemented with 5% calf serum, penicillin (50 U/ml), and streptomycin (50 μg/ml). Cells were seeded at a density of 2.2×10^5 cells/ml/16-mm well on 24-well plates (Falcon Plastics, Cockeysville, MD; Costar Corp., Cambridge, MA) coated, as described below, with either gelatin (ICN Biomedicals, Costa Mesa, CA) or matrix prepared from either cardiac fibroblasts (used at first passage after the differential plating) or NRK rat kidney fibroblasts.

Fibroblast matrix was prepared as previously described (21). Briefly, confluent monolayers of either cardiac or NRK fibroblasts were washed twice in PBS (without Ca^{++} and Mg^{++}) and then incubated in the same plus 5 mM EDTA (pH 7.2), at 37°C for 45 min to release the cells. After washing in the releasing solution, wells were washed twice in regular PBS, and stored in PBS at 0°C for up to 4 wk. For gelatin coating, a 0.1% gelatin solution was added to the wells for 1 h. Before plating the myocytes, plates were washed one additional time with PBS.

Measurement of the beating rate of the cultured myocytes. Myocytes were fed fresh medium containing 5% calf serum 48 h after plating. 72 h after plating, at which time the myocytes have formed a syncytium and beat synchronously, they were washed with serum-free DMEM/F12 containing insulin, transferrin, selenium, linoleic acid, and bovine serum albumin (ITS+; Collaborative Research, Waltham, MA) for 30 min, and then placed in fresh serum-free medium containing the added growth factors (time 0). Beating rate in beats per minute (bpm) was measured by counting the time required for 30 beats of the myocytes using an enclosed, heated stage maintained at 37°C (NP-2 incubator; Nikon Inc., Melville, NY). In any particular experiment, beating rate was usually measured at time points ranging from 2 to 48 h after the switch to serum-free conditions (starting 72 h after preparation of the myocytes, as described above) and averaged for 4–6 replicate wells per treatment.

Quantitation of TGF-β secreted from cultured myocytes. Medium conditioned by cultured cardiac myocytes was collected into tubes containing the protease inhibitors aprotinin, leupeptin, and pepstatin A (each at 1 μg/ml final concentration) and frozen at −20°C until assayed. Total TGF-β in the medium was quantitated as described previously by comparison of the activity to that of a TGF-β standard in the mink lung cell CCL-64 growth inhibition assay (17). Specific antibodies were used in the assay to determine the relative contributions of the different TGF-β isoforms.

Cross-linking of TGF-β to cell surface proteins. Either cardiac fibroblasts used at the first passage or freshly prepared purified myocytes were plated at a density of 1×10^6 cells per 35-mm dish coated with gelatin and cultured for 72 h in media containing 5% calf serum. At this point, cells were washed with binding buffer (DMEM, 25 mM Hepes, 1 mg/ml BSA, pH 7.4) and cross-linked to the iodinated TGF-β using disuccinimidyl suberate (Pierce Chemical Co., Rockford, IL) as previously described (22).

Results

Initial studies using crude preparations of myocytes cultured in medium containing 5% serum on plates coated with gelatin demonstrated that IL-1β (0.3 to 5 ng/ml, added at $t = 0$), suppressed the beating rate of the cells measured 24 h later; this inhibition was overcome by the addition of TGF-β1 (5 ng/ml). TGF-β1 was equally effective whether it was added 18 h before or simultaneously with the IL-1β (not shown).

Since these cultures contained variable numbers of cells other than myocytes, we purified the myocytes further on Percoll gradients (Sigma Chemical Co.) (19, 20). Moreover, to characterize the nature of the response more precisely, myocytes were switched to a defined serum-free medium 72 h after the initial plating. At the time of changeover to serum-free conditions with appropriate growth factor additions ($t = 0$), the neonatal myocytes were subconfluent and were beating strongly and synchronously, usually at rates ranging from 120 to 150 bpm. In contrast to our earlier results, culture of these purified myocytes on wells coated with gelatin showed only minor effects of the added growth factors (Fig. 1, *middle*); however, when these same myocytes were cultured together with cardiac fibroblasts, in an attempt to mimic the initial experiments with the unfractionated myocytes, the suppressive

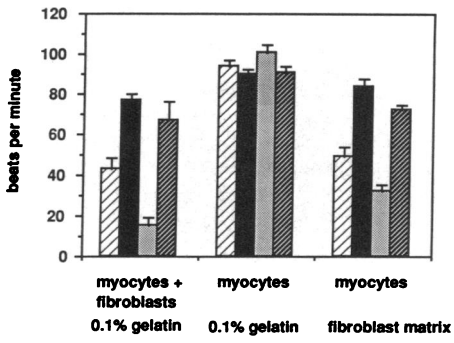

Figure 1. Effects of TGF-β and IL-1β on the beating rate of cultured neonatal myocytes. Myocytes were fractionated on Percoll gradients as described and cultured in serum-free DMEM/F12 ITS+ either together with 0.5×10^5 cardiac fibroblasts (*left*) or alone (*middle, right*) on wells coated either with 0.1% gelatin (*left, middle*) or with matrix prepared from cardiac fibroblasts as described in Methods (*right*). Data represent the mean±SEM of the beating rate at 24 h of 12 replicate wells in 2 separate experiments treated at the time of the down-shift to serum-free medium with vehicle alone (▨), TGF-β1, 5 ng/ml (■), IL-1β, 5 ng/ml (□), or the combination of TGF-β1 and IL-1β (■).

effects of IL-1β and the protective effects of TGF-β1 were once again observed (Fig. 1, *left*).

To ascertain whether both cultured neonatal cardiac myocytes and fibroblasts displayed cell surface receptors for TGF-β, iodinated TGF-βs were cross-linked to each of these cell populations, the cells were lysed, and the pattern of binding examined after electrophoresis on SDS gels. As shown in Fig. 2, both myocytes and fibroblasts displayed the characteristic pattern of cross-linking of TGF-β to receptors, showing all three types of binding proteins (23, 24). Although the relative proportions of the presumed signaling receptors (types I and II) to that of the cell-surface proteoglycan (type III) varied for the two cell types, each cell type appeared to bind all three TGF-β isoforms similarly. These data suggest that cardiac myocytes and fibroblasts both express cellular receptors for TGF-β.

We next showed that the effects of coculture of myocytes and fibroblasts could be reproduced by culture of the purified myocytes on matrix secreted by either cardiac fibroblasts (Fig. 1, *right*) or by NRK fibroblasts (data not shown), but not by simple addition of medium conditioned by cardiac fibroblasts exposed to the growth factor regimens (data not shown). Culture of the myocytes on purified laminin or fibronectin gave a pattern similar to that shown for myocytes cultured on gelatin (data not shown). These data demonstrate that the response of the cultured myocytes to cytokines can be modulated by the matrix on which they are grown, independent of direct cell-to-cell contact between myocytes and fibroblasts. At the present time, it is not known whether these effects are mediated by cytokines or other bioactive factors sequestered in the fibroblast matrix, or whether they might result from subtle cytoskeletal and phenotypic changes in the myocytes after attachment to either gelatin or the native fibroblast matrix.

Both the actual beating rate and its rate of decline with time were dependent on the concentration of added TGF-β1, when myocytes were cultured in serum-free medium on fibroblast matrix. As shown in Fig. 3, at any particular time, the beating rate was dependent on the concentration of added TGF-β1; concentrations of TGF-β1 as low as 3 pM (78 pg/ml) still enhanced the beating rate of the myocytes relative to control. In no case did the addition of TGF-β1 enhance the beating rate relative to the initial rate at the time of change of the cells to serum-free conditions. Rather, TGF-β1 maintained the initial rate or lessened its decline during extended culture under serum-free conditions. Thus, myocytes cultured in the presence of TGF-β1 at concentrations greater than 1 ng/ml were still beating at more than 50% their initial rate 40 h after the onset of serum-free conditions, in contrast to control cultures, in which beating had stopped entirely (Fig. 3). After 72 h, 5/5 and 4/5 cultures of myocytes at TGF-β1 concentrations of 5 and 1.2 ng/ml, respectively, were still beating (data not shown). In contrast, myocytes cultured in the absence of TGF-β1 ceased to beat after approximately 24 h culture in serum-free medium (Fig. 3). However, these myocytes maintained their capacity to beat, since beating was restored upon addition of serum (not shown). Each of the three TGF-β isoforms had equivalent stimulatory effects on the beating rate of the myocytes at 5 ng/ml (data not shown), although a complete dose-response analysis for TGF-βs 2 and 3 was not performed.

Significantly, careful observation of the cultures demonstrated not only that TGF-β1 maintained the beating rate of myocytes cultured on fibroblast matrix, but also that myocytes cultured in the presence of TGF-β1 generally had a more regular beating pattern than that of cells cultured in its absence. As shown in Table I, over 50% and 75% of the control or IL-1β-treated myocyte cultures, respectively, were characterized by an irregular beating pattern, and an additional 11% and 25% of the same cultures, respectively, had either stopped beating or were beating at a rate greater than 200 bpm ("fibrillating"). In contrast, only 5% and 20% of the cultures treated with TGF-β1 alone or TGF-β1 plus IL-1β, respectively, had an irregular beating pattern, and no cultures in either of these groups had either stopped beating or were fibrillating.

To examine whether myocytes might be secreting factors capable of modulating their beating rate, cells were cultured

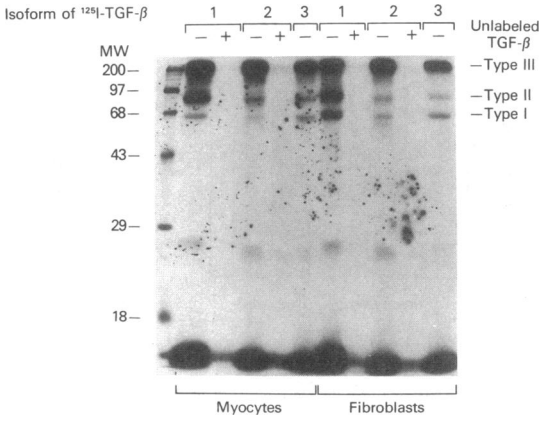

Figure 2. Receptor cross-linking to ^{125}I-TGF-β. Purified myocytes (*left*) or cardiac fibroblasts after first passage (*right*) were cross-linked to either ^{125}I-labeled TGF-β1, 2, or 3 in the presence or absence of 100-fold excess of unlabeled TGF-β (TGF-β1 and 2 only). Extracts were loaded onto a 10% polyacrylamide gel, electrophoresed, and the dried gel was exposed to film for 1–2 wk. Molecular mass markers are indicated on the left. The receptor types are indicated on the right showing the type I receptor (65 kD), the type II receptor (85 kD), and the high molecular mass type III receptor (>200 kD). Free monomeric ^{125}I-TGF-β can be seen at the bottom of the gel.

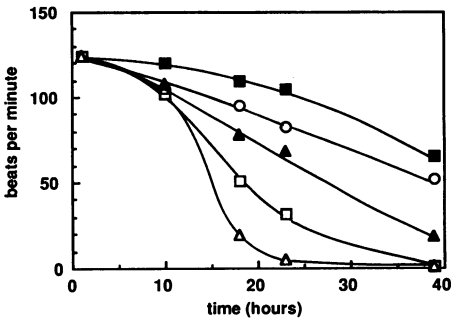

Figure 3. Effects of varying concentrations of TGF-β on the beating rate of myocytes cultured on cardiac fibroblast matrix in serum-free ITS+ medium. Myocytes were purified on a Percoll gradient and plated on cardiac fibroblast matrix as described. Cells were then incubated in serum-free DMEM/F12 containing insulin, transferrin, selenium, linoleic acid, and bovine serum albumin in the presence or absence of TGF-β1 at the concentrations indicated: 5 ng/ml (■); 1.2 ng/ml (○); 0.31 ng/ml (▲); and 0.078 ng/ml (□); none (△). Data are the average of five replicate wells for each time point in a representative experiment; two replicate experiments gave similar results.

Table I. Effects of IL-1 and TGF-β on the Rate and Pattern of Beating of Myocytes Cultured in Serum-Free Medium Supplemented with ITS+

Treatment	Average bpm±SEM	Irregular beating (number per 44 wells)	Fibrillating or stopped (number per 44 wells)
24 h			
Control	67±5.6	24	5
TGF-β	98±4.4	2	0
IL-1	37±4.2	33	11
IL-1 + TGF-β	81±5.8	9	0

Beating rate was measured 24 h after addition of either TGF-β, IL-1β, or the combination of the two, each at 5 ng/ml. Data represent the mean beats per minute (bpm)±SEM of a summation of five experiments with a total of 44 wells in each treatment group. The irregular beating pattern of the IL-1β-treated or control cultures was most often characterized as a series of beats (average 2–10) followed by a pause of duration 2–20 s in which there was no beating; for a given culture, this pattern was often quite reproducible. The contrast of this pattern with the regular consistent beating (no pauses) observed in cultures treated with TGF-β is easily discernable to any observer.

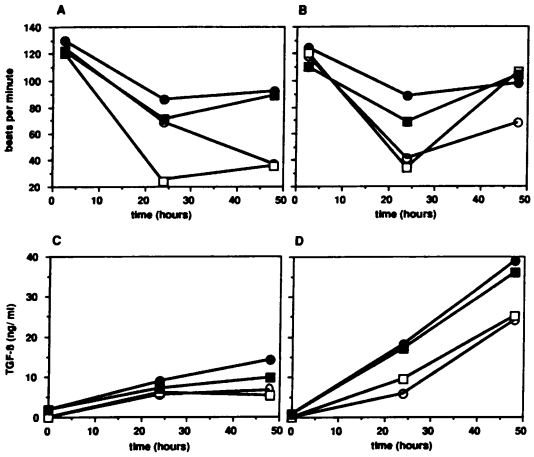

Figure 4. Effects of reduced medium volume on the beating rate and TGF-β secretion of myocytes. Myocytes purified on Percoll gradients were cultured in wells coated with cardiac fibroblast matrix. At the start of the experiment, either 1 ml (*A, C*) or 0.25 ml (*B, D*) serum-free ITS+ medium containing vehicle alone (○), TGF-β1, 5 ng/ml (●), IL-1β, 5 ng/ml (□), or TGF-β1 plus IL-1β (■) was added to each 16-mm well. (*A, B*) The change in the beating rate of the myocytes with time in the two different volume conditions. At 48 h, the difference in beating rates of IL-1-treated cultures in the high and low volume conditions is significant at the $P = 0.001$ level, using a standard Student's two-tailed t test. (*C, D*) The concentration of TGF-β (total, active plus latent) in the medium was determined in a growth inhibition assay using CCL-64 cells as described in Methods. Results represent the average of data from 16 wells combined from three separate experiments.

under reduced volume conditions where any secreted factors would be more concentrated. Experiments in which equal numbers of myocytes were cultured identically on fibroblast matrix for the first 72 h and then shifted at $t = 0$ to either 1 ml or 0.25 ml of serum-free medium, showed that the beating rate of cells cultured in the absence of added TGF-β1 (control or IL-1 treated) under either of these two conditions decreased similarly during the first 24 h of culture. However, during the next 24-h period, the beating rate of those myocytes treated with IL-1 and cultured in 0.25 ml, but not the standard 1-ml vol of medium, increased significantly ($P = 0.001$, Fig. 4, *A* and *B*). In contrast the beating rates of myocytes cultured in the presence of added TGF-β1 were not appreciably affected by reduction of the volume of medium, over the same observation period. One possible explanation for these data is that stimulatory factors might be accumulating in the medium over time in culture.

Since TGF-β1 appeared to replace the need for the putative endogenous factor, we examined whether TGF-β might be secreted by the myocytes and whether an autocrine response to TGF-β might play a role in maintaining the beating rate of the cells. As shown in Fig. 4,*C* and *D*, analysis of the total TGF-β secreted from the cells (all three isoforms, after activation) demonstrated both that the myocytes secreted relatively high levels of TGF-β in the range of 5–10 ng/ml in the 1-ml cultures, to as high as four times that amount in the 0.25-ml cultures, and that the secretion of TGF-β was autostimulated to a small extent by culture of the cells in the presence of TGF-β1. Analysis of latent vs. active TGF-β demonstrated that the myocytes secreted TGF-β principally in the latent form and that detectable amounts of active TGF-β could be found only in those cultures to which it had been added. Isotyping using antibodies specific for either TGF-βs 1 or 2 showed that 70–85% of the TGF-β secreted by the myocyte cultures is TGF-β2 (data not shown).

The above experiments suggested that there might be a threshold concentration of TGF-β necessary to maintain the beating rate of the myocytes, which was met or exceeded only after extended culture in the more concentrated condition of the low volume cultures. A corollary would be that removal of TGF-β from the medium should suppress the intrinsic beating rate and accelerate the rate of decline of the beating rate. Indeed, as shown in Fig. 5, addition of a monoclonal antibody that blocks the activity of TGF-βs 1, 2, and 3 markedly acceler-

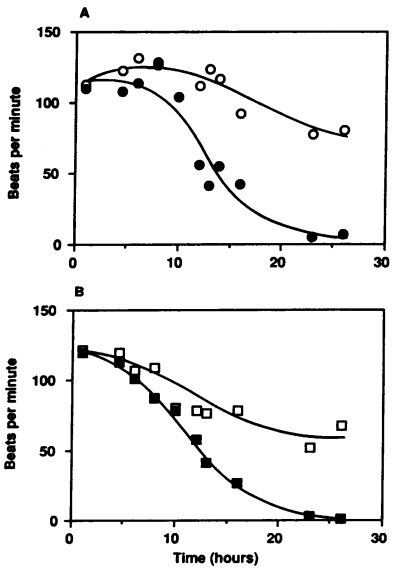

Figure 5. Effects of a monoclonal antibody to TGF-β on the beating rate of myocytes. Myocytes were shifted to serum-free medium supplemented with ITS+ and containing either 14 μg/ml of a purified murine IgG1 monoclonal antibody to TGF-β (●, ■) or the identical concentration of purified murine ascites fluid IgG1 (○, □) in the absence (*A*) or presence (*B*) of IL-1β, 5 ng/ml. The data show a representative experiment of five similar experiments; each point is the average of six replicate wells.

Table II. Effects of Different Cytokines on the Beating Rate of Myocytes in the Presence or Absence of IL-1

Cytokine*	Beats per minute of myocytes‡ (mean±SEM)			
	Without IL-1β		With IL-1β	
	14 h	*19 h*	*14 h*	*19 h*
TGF-β	133±13	95±8	114±7.9	90±8
PDGF-BB	61±4	16±7	65±6.3	26±4.5
bFGF	41±14	2±2	27±4.9	15±3.1
EGF	15±4	1±1	20±3.1	7±2.7
None	7±2	0	18±6.0	7±2.5

* Each cytokine (including IL-1β) was added at time 0 at an initial concentration of 5 ng/ml. The biological activity of the peptides was confirmed in a growth assay using NRK fibroblasts. In this assay, cell number increased 5.7, 3.7, and 2.3-fold over control in cultures grown for 5 d in 1% serum supplemented with 5 ng/ml EGF, PDGF, or bFGF, respectively; TGF-β inhibited the growth of the cells with cell numbers being only 43% of control after 5 d in culture. ‡ Beating rate of myocytes was measured at the indicated time after cytokine addition; initial beating rate was 138 bpm. Results represent the mean±SEM of 4–5 replicate wells in a single experiment representative of three additional experiments of identical design.

ates the decline of the beating rate of the cells, clearly demonstrating that endogenous TGF-β secreted by the cultured cells contributes to their intrinsic ability to beat in culture. The rate of decline of the beating in the presence of the antibody was similar in the presence or absence of IL-1, suggesting that one of the actions of IL-1 might be to antagonize the autocrine activity of the TGF-β secreted by the myocytes. A similar pattern of inhibition (not shown) was observed using a combination of polyclonal antibodies specific for TGF-β1 (turkey) and 2 (rabbit) compared to appropriate control sera.

To investigate whether TGF-β was unique in promoting these observed effects, three other growth factors, namely PDGF BB, bFGF, and EGF, were assayed in a similar experimental design to determine their efficacy both in maintaining the basal beating rate of the myocytes in serum-free medium and in antagonizing the effects of IL-1β (see Table II). At early times, the results were somewhat variable from experiment to experiment, with PDGF BB and bFGF having a moderate positive effect on the beating rate. However, without exception, only those cultures treated with TGF-β were able to sustain a regular beating pattern after 19 to 48 h of culture. Relative effects of each of these growth factors were similar in the presence or absence of added IL-1. Thus, within this set of factors, TGF-β is unique in its ability to sustain rhythmic beating of the cultured myocytes for an extended period of time in serum-free culture.

Discussion

In these experiments, we have demonstrated for the first time that exogenous TGF-β is uniquely able to regulate the beating rate of cultured neonatal rat myocytes, both maintaining the regular rhythm and the rate of beating of cells cultured in serum-free medium. More importantly, we have demonstrated that cultured myocytes secrete relatively high levels of TGF-β and that this endogenous TGF-β acts in an autocrine fashion to sustain the beating rate of myocytes cultured in serum-free medium. The data are consistent with our previous proposals that TGF-β might accelerate repair and restore function to myocytes which stain intensely for TGF-β1 and are located at the margin of an infarcted area of the heart (2).

Our results also confirm that IL-1 suppresses myocyte contractility (10) and show for the first time that TGF-β can antagonize its suppressive effects on the beating rate. Previous studies have shown that supernatants of activated immune cells inhibit the responsiveness of neonatal myocytes to β-adrenergic stimulation in terms of both a contractile response and intracellular cAMP accumulation (10); the immune cell secretory products, IL-1 and tumor necrosis factor-α, had similar effects. The specific mechanisms involved in the antagonistic effects of TGF-β and IL-1β on the beating rate of neonatal myocytes are not known, although TGF-β opposes the actions of IL-1 and tumor necrosis factor in a variety of in vitro systems, and the mechanisms are understood in some systems (11–14, 23, 25). Based on these investigations, possible mechanisms might include downregulation by TGF-β of IL-1 receptor expression, as demonstrated in lymphoid and myeloid progenitors (26) and chondrocytes (27), or upregulation by TGF-β of expression of the IL-1 receptor antagonist as demonstrated in peripheral blood monocytes (28). At a different level, TGF-β has been shown to increase matrix protein synthesis and to decrease protease secretion (29), while the corresponding effects of IL-1 are exactly opposite (30). The rather prolonged time frame of our observed effects of these cytokines on myocyte function is consistent with any of these mechanisms.

TGF-β administered systemically after experimental infarction has been shown to have cardioprotective effects (31, 32). Similar protective effects of TGF-β have been demonstrated in a model of splanchnic ischemia-reperfusion injury (32). One of the effects of systemic TGF-β administration is to suppress release of IL-1 and TNFα into the circulation after reperfusion. However, these effects have been suggested to be mediated indirectly via the ability of TGF-β to preserve and stabilize endothelial function and to inhibit endothelial adhesiveness for neutrophils (32, 33). This mechanism therefore is distinct from the direct antagonism by TGF-β of the effects of IL-1β on cardiac myocytes that we have described here.

Our data leave open the question of the mechanism of action of TGF-β on the myocytes, since preliminary attempts to identify such mechanisms have been unsuccessful. Thus, measurement of cellular ATP levels showed no significant differences whether myocytes were treated with TGF-β or with IL-1β (data not shown). Moreover, changes in cAMP levels in response to treatment with isoproterenol (10) were suppressed equivalently relative to control in myocytes treated with either TGF-β or IL-1β (data not shown), suggesting that changes in cAMP could not directly be related to the observed opposite effects of TGF-β and IL-1 on the beating rate of the myocytes. Likewise, since both TGF-β and bFGF have similar effects on expression of α and β-myosin heavy chain genes by these cells (1, 18), but different effects on their beating rates (Table II), effects of these two cytokines on differentiation cannot explain the observations reported here. Lastly, investigations into possible effects of TGF-β on regulation of calcium in myocytes demonstrated that whereas culture of myocytes in low calcium concentrations (0.2 to 1 mM) suppressed the beating rate of the

cultures in a concentration-dependent manner, the suppression could not be overcome by TGF-β added either 18 h previous to or at the same time as the downshift of the calcium (data not shown).

The ability of matrix to regulate the actions of cytokines on cell phenotype and cell function has been observed in numerous in vitro systems (34). For example, type IV collagen, fibronectin, and laminin coatings differentially affect the growth of cultured endothelial cells, and the inhibition of the growth of these cells by TGF-β is thought to be mediated, in part, by its ability to enhance cellular fibronectin secretion (35). Moreover, both TGF-β1 and IL-1β have been shown to regulate expression of cell adhesion integrin receptors on a variety of cell types (36–38). If these cytokines were able to modulate differentially integrin receptor expression on neonatal myocytes, it could subtly affect the shape or spreading, and consequently the contractility, of myocytes cultured on the complex, native fibroblast matrix compared to gelatin (principally denatured type I collagen), laminin, or fibronectin. In addition, cytokines such as TGF-β (39, 40) and FGF (41, 42) can bind to proteoglycan matrix components. Whether the altered contractile response patterns to exogenous cytokines of myocytes cultured on a more homogeneous, defined matrix compared to a native fibroblast matrix (Fig. 1) might result from effects of these or other unknown sequestered cytokines cannot be determined at the present time. Although it has not escaped our attention that disorders of the collagen matrix result in compromised mechanical properties of the heart (43–45), the relationship of our in vitro model system to that of a complex organ in vivo is unclear.

Future directions of this research will be focused on characterizing specific roles of the three TGF-β isoforms in both basal function of cardiac myocytes and their response to injury and stress. Moreover, we hope to identify the mechanisms of action of TGF-β and IL-1 on myocytes, including the modulating role of the matrix, with the ultimate goal of increasing our understanding of the role of these cytokines in the clinical setting of the response of the heart to both acute and chronic injury.

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