Targeted Expression of Transforming Growth Factor-β1 in Intracardiac Grafts Promotes Vascular Endothelial Cell DNA Synthesis

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Abstract

Intracardiac grafts comprised of genetically modified skeletal myoblasts were assessed for their ability to effect long-term delivery of recombinant transforming growth factor-β (TGF-β) to the heart. C2C12 myoblasts were stably transfected with a construct comprised of an inducible metallothionein promoter fused to a modified TGF-β1 cDNA. When cultured in medium supplemented with zinc sulfate, cells carrying this transgene constitutively secrete active TGF-β1. These genetically modified myoblasts were used to produce intracardiac grafts in syngeneic C3Heb/FeJ hosts. Viable grafts were observed as long as three months after implantation, and immunohistological analyses of mice maintained on water supplemented with zinc sulfate revealed the presence of grafted cells which stably expressed TGF-β1. Regions of apparent neovascularization, as evidenced by tritiated thymidine incorporation into vascular endothelial cells, were observed in the myocardium which bordered grafts expressing TGF-β1. The extent of vascular endothelial cell DNA synthesis could be modulated by altering dietary zinc. Similar effects on the vascular endothelial cells were not seen in mice with grafts comprised of non-transfected cells. This study indicates that genetically modified skeletal myoblast grafts can be used to effect the local, long-term delivery of recombinant molecules to the heart. (*J. Clin. Invest.* 1995. 95:114–121.) Key words: C2C12 skeletal myoblasts • intracardiac grafts • cell transplantation • gene therapy • myocardial repair • angiogenesis

Introduction

Cellular grafting has emerged as an accepted approach for the delivery of therapeutic agents to patients. For example, intracerebral grafting of fetal adrenal tissue in Parkinson's patients has gained support as an effective means to supplement dopamine levels (for review see reference 1). More recently, the use of genetically modified cells for the delivery of recombinant molecules has emerged as a powerful approach for ex vivo gene therapy. Several experimental animal models which use a variety of cell types to deliver recombinant molecules to the brain, thymus, and hematopoietic system have recently appeared (for review see references 2–5). Using an analogous approach in humans, autologous transplantation of bone marrow cells transfected with an adenosine deaminase expression construct has been used in an attempt to treat patients lacking this enzyme (6). More recently, ex vivo gene therapy with autologous hepatocytes has been successfully used to target expression of recombinant LDL receptor in a patient with familial hypercholesterolaemia, resulting in an improved plasma LDL/HDL ratio (7).

Skeletal myoblasts possess several attributes which have proven to be beneficial for grafting experiments, particularly into muscle beds. For example, primary myoblasts can be isolated from numerous species including humans; the use of autologous grafts comprised of amplified primary myoblasts could eliminate immune rejection. In addition, skeletal myoblasts are genetically tractable, thereby allowing the introduction of recombinant genes in vitro. When engrafted into the skeletal muscle beds of *mdx* mice, primary myoblasts from normal mice differentiated and fused with diseased host myotubes (8). The resulting heterokaryons expressed dystrophin. Heterokaryon formation in these models was dependent upon the ability of myoblasts to traverse the basal lamina of pre-existing myotubes (9). These later observations suggested that myoblast grafting might constitute a potential therapeutic intervention for Duchenne's patients. Although initial efforts to treat patients with this approach appear to have attained some degree of success in that a limited number of dystrophin-positive cells could be detected in muscle biopsies from engrafted patients (10), the procedure is not without controversy (11).

Genetically modified skeletal myoblast grafts have been successfully used to deliver recombinant proteins in experimental animals. Stably transfected myoblasts injected intramuscularly into the limbs of mice effectively elevated plasma growth hormone levels for up to 80 d after grafting (12, 13). In more recent studies primary myoblasts transfected with a tyrosine hydroxylase expression construct have been engrafted into the brain of rats with unilateral striatal denervation (14). Dopamine and L-DOPA secretion from the grafted myocytes correlated inversely with rotational behavior in this surgical model of Parkinson's disease.

The use of intracardiac grafting to effect long-term delivery of recombinant proteins could induce a beneficial response in the myocardium. For example, grafts expressing angiogenic factors might induce neovascularization. Grafts expressing neurotrophic agents near an infarcted region might ameliorate the arrhythmogenesis associated with the border zone (15). Given the potential therapeutic value of long-term recombinant protein delivery in the heart, we initiated a series of experiments aimed at identifying cell types capable of forming stable grafts in the myocardium. Our initial study used AT-1 cells, a differentiated cardiomyocyte tumor line derived from transgenic mice which express the SV40 large T antigen oncogene in the atrium (for review see reference 16). AT-1 cardiomyocytes formed long-term, differentiated intracardiac grafts when introduced into the myocardium of syngeneic animals (17). Moreover, no deleteri-

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ous effects on host cardiac function were observed. Unfortunately, because AT-1 cardiomyocytes are transformed, their utility for producing long-term intracardiac grafts is somewhat limited. Subsequent studies utilized C2C12 myoblasts to form intracardiac grafts (18). Grafted myoblasts differentiated and fused to form nascent myotubes within the host myocardium. Furthermore, grafted C2C12 myoblasts withdrew from the cell cycle upon myodifferentiation; consequently, proliferation of graft myocytes was not an issue with this model.

The present study examined the capacity of genetically engineered myoblasts to effect long term delivery of recombinant protein to the heart. C2C12 myoblasts were stably transfected with a fusion gene comprised of the metallothionein promoter and a TGF-β1 cDNA. TGF-β1's activity as an angiogenic factor in vivo is well characterized (19). Moreover, it has recently been suggested that TGF-β1 exhibits cardioprotective properties (20). Recombinant TGF-β1 expression was readily detected in grafts generated with modified C2C12 myoblasts, but not in control grafts. Furthermore, evidence for enhanced neovascularization was observed in the myocardium bordering grafts expressing TGF-β1. The implication of these results with respect to the potential long-term delivery of recombinant proteins to the heart is discussed.

Methods

C2C12 cell culture and transfection. C2C12 myoblasts (American Type Culture Collection, Rockville, MD) were maintained in the undifferentiated state by culturing at low density in high glucose DME supplemented with 20% fetal bovine serum, 1% chicken embryo extract, 100 U/ml penicillin, and 100 μg/ml streptomycin. For some studies, myogenic differentiation was induced by culturing in DME supplemented with 2% horse serum and antibiotics.

Transfections utilized pPK9a, a TGF-β1 expression vector under the control of the metallothionein promoter, kindly provided by P. Kondaiah (21). pPK9a was stably cotransfected with the neomycin resistance gene into C2C12 cells by the calcium phosphate precipitation method. Neomycin-resistant colonies were screened for expression of the recombinant TGF-β1 transcripts in the presence of 100 μM zinc sulfate.

Myocardial grafting protocol. Immediately before injection, transfected and control myoblasts were harvested with trypsin, washed three times with serum-free DME, and directly injected into the ventricular myocardium of adult syngeneic C3Heb/FeJ mice (Jackson Laboratories, Bar Harbor MA) under open heart surgery as described (17, 18). 4–10 x 10^4 cells were injected in a volume of 2–3 μl using a plastic syringe fitted with a 30-gauge needle. 1 mo after surgery, graft bearing animals were given heavy metal (25 mM zinc sulfate in drinking water), and treatment was continued until the termination of the experiment (7–50 d).

Northern blot analysis. Cells were plated onto 150-mm tissue plates. At 70–80% confluence, TGF-β1 secretion was induced by 100 μM zinc sulfate. After 24 h of incubation, total RNA was isolated as described (22). 10 μg of each sample of total RNA was run on a 1.0% agarose, 1.1 M formaldehyde gel, and blotted onto a Duralon-UVT^M membrane (Stratagene, La Jolla, CA). Prehybridization, hybridization, and washing of the filters were as described (23). RNA blots were hybridized with probes corresponding to the rat TGF-β1 cDNA (24) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH)[1] cDNA (25).

Preparation of conditioned medium and CCL64 mink lung growth inhibition assay. Cell lines were cultured in 100-mm tissue culture plates in high glucose DME supplemented with 20% fetal bovine serum. At 70–80% confluency, cells were washed once with serum-free medium and then cultured in DME supplemented with 100 μg/ml BSA fraction V for 24 h with or without either 50 or 100 μM zinc sulfate. Conditioned media were centrifuged, and then 1 μg/ml aprotinin, 2 μg/ml leupeptin, and 120 μg/ml PMSF were added. The amount of TGF-β being secreted into the medium was determined by the CCL64 growth inhibition assay as described (26). TGF-β monoclonal antibody (Genzyme Corp., Cambridge, MA) which neutralizes all three TGF-β forms (TGF-β1, TGF-β2, and TGF-β3) was used to block the biological activity of TGF-βs.

Histology. For paraffin sections, hearts were initially fixed in 10% neutral buffered formalin followed by Bouin's fixative, dehydrated through graded alcohols, and infiltrated with paraffin under standard procedures. Tissue blocks were then sectioned at 6 μm. Hematoxylin and eosin (H and E) staining was performed directly after deparaffination according to manufacturer's specifications (Sigma Diagnostics, St. Louis, MO). Immunohistologic analysis of TGF-β1 expression was as described (27). Briefly, deparaffinized sections were hydrated and blocked with phosphate buffered saline containing 1% BSA and 5% goat serum. Sections were then incubated with anti-TGF-β1 antibody (CC-1–30–1), and signal developed with an avidin-biotin (ABC) kit (Vector Laboratories, Inc., Burlingame, CA). For [^{3}H]thymidine incorporation, mice were given a single injection of isotope (400 μCi at 28 Ci/mM; Amersham, Arlington Heights, IL) and sacrificed 14 h later. The heart was removed and processed for paraffin embedding as described above. For autoradiography, deparaffinized sections were stained with H and E, and coated with a thin layer of photographic emulsion (Ilford L.4; Polysciences Inc., Warrington, PA) diluted 1:1 with distilled water. Sections were exposed for 5–7 d at 4C, and developed in Kodak D-19 (Eastman Kodak Co., Rochester, NY) at 20C for 4 min, washed with distilled water for 1 min, fixed in 30% sodium thiosulfate for 10 min, and washed in distilled water. Immunohistologic analyses with anti-von Willebrand Factor (vWf) antibody (Dako Corp., Carpinteria, CA) was used to localize vascular endothelial cells according to the supplier's recommendations. Monoclonal antibody against the common leukocyte antigen (CD25; antibody M1/9.3HL; Boehringer Mannheim, Indianapolis, IN) was used to monitor immune rejection of the targeted recombinant TGF-β1. This antibody recognizes all lymphoid and hematopoietic cells, including B and T lymphocytes, monocytes, and granulocytes. After treatment with primary antibody, sections were incubated with horseradish peroxidase-conjugated rabbit anti-rat antisera (Boehringer Mannheim), and visualized by the ABC kit according to the manufacturer's protocol (Vector Laboratories, Inc.).

Assessment of myocardial vascular endothelial cell DNA synthesis. To quantitate vascular endothelial DNA synthesis, samples were monitored via a Sony CCD television camera mounted on a Leitz Labrolux microscope, and images were displayed on a video monitor. The number of synthetic vascular endothelial cells, as evidenced by tritiated thymidine incorporation, which resided within 500 μm of the graft/myocardium border were counted. The cumulative length of the graft/myocardium border for each experimental series was also determined. Results are expressed as the ratio of the total number of synthetic vascular endothelial cells divided by the cumulative length (arbitrary units) of graft/myocardium border. Data is presented as means ± standard error. A nested group *t* test was used for statistical analyses.

Results

A fusion gene comprised of the metallothionein promoter driving a modified TGF-β1 cDNA was described previously (21). Transcriptional activity of the metallothionein promoter can be regulated by modulating the heavy metal content of cell culture medium. The TGF-β1 cDNA carried site-directed mutations which resulted in the conversion of cysteine^{223} and cysteine^{225} to serines. This modification (28) results in the elaboration of a TGF-β1 molecule in which the portion of the prepro domain which is processed to the latency associated protein (LAP) is unable to form dimers, and consequently cannot confer latency

1. *Abbreviations used in this paper:* FGF, fibroblast growth factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase, H and E, hematoxylin and eosin; VEGF, vascular endothelial growth factor.

on mature TGF-β. Cells expressing the modified cDNA constitutively secreted active TGF-β1 (21). The metallothionein-TGF-β1 fusion gene was introduced into C2C12 myoblasts by calcium phosphate transfection; stable transfectants were selected by virtue of co-transfection with an SV40-neo^r transgene. Four independent clones were isolated by this approach, and presence of the transgene was confirmed by Southern blot analysis (not shown). The relative levels of TGF-β1 expression in the different clonal cell lines was initially assessed by Northern blot analysis, and one line, designated C2(280), was selected for subsequent experiments.

Expression of recombinant TGF-β1 in response to heavy metal induction was examined in C2(280) myoblasts and myotubes. Transgene transcripts (1.8 kb) were readily distinguished from those originating from the endogenous TGF-β1 gene (2.5 kb) by Northern blot analysis (Fig. 1 *A*). Addition of heavy metal to the culture medium resulted in a marked increase of recombinant TGF-β1 transcripts in C2(280) myoblasts and myotubes. As indicated above, modified TGF-β1 expressed by C2(280) cells should have constitutive biological activity. To directly test this, conditioned medium from C2(280) myoblasts and myotubes was examined by the CCL-64 growth inhibition assay (26). When induced, C2(280) cells secreted TGF-β at a rate of 31 ng/10^6 cells/24 h, 9.6-fold more than in the uninduced state (Fig. 1 *B*). Furthermore, after zinc sulfate treatment, a very high proportion of the TGF-β (49%) was active in neutral conditioned medium and did not require acid activation.

C2(280) myoblasts were used to produce intracardiac grafts in syngeneic C3Heb/FeJ mice. The presence of grafts was readily detected in H and E stained sections (Fig. 2 *A*). 100% of the animals receiving intracardiac injections of C2(280) cells went on to develop grafts. Interestingly, H and E analysis suggested that the C2(280) grafts were somewhat less differentiated as compared with those produced with unmodified C2C12 cells (compare Fig. 2, *A* and *C*; see also reference 18, Discussion). The differentiated state of the engrafted C2(280) cells was further explored by immunohistologic analysis with a monoclonal antibody specific for skeletal myosin heavy chain; nascent myotubes appeared somewhat disorganized in C2(280) grafts as compared with control C2C12 grafts (data not shown).

Graft transgene expression was assessed by immunohistology with an anti-TGF-β1 antibody (27). TGF-β1 expression was readily detected in C2(280) grafts when the host animal was maintained on zinc supplemented water (Fig. 2 *B*). Furthermore, removal of zinc resulted in a marked decrease in graft TGF-β1 expression (data not shown). As a negative control, TGF-β1 expression was assessed in grafts produced by untransfected C2C12 myoblasts (Fig. 2 *D*). As expected, TGF-β1 expression was virtually undetectable in the C2C12 grafts (Fig. 2 *D*). Although surface electrocardiograms of either C2C12 or C2(280) engrafted mice were normal, the relatively high heart rate in mice might render these animals resistant to experimentally induced arrhythmia. As such, potential arrhythmogenic effects of chronic TGF-β1 delivery might not be detected in this model.

Given that TGF-β1 is a well known angiogenic factor (19), several experiments were initiated to determine if augmented blood vessel growth could be detected in the myocardium bordering C2(280) grafts. The initial study employed immunohistological analysis of vWf expression. vWf is a well-established marker for vascular endothelial cells; assessment of vWf immunoreactivity consistently revealed a marked tendency towards greater capillary density in the myocardium bordering the TGF-β1 expressing C2(280) grafts as compared to control C2C12

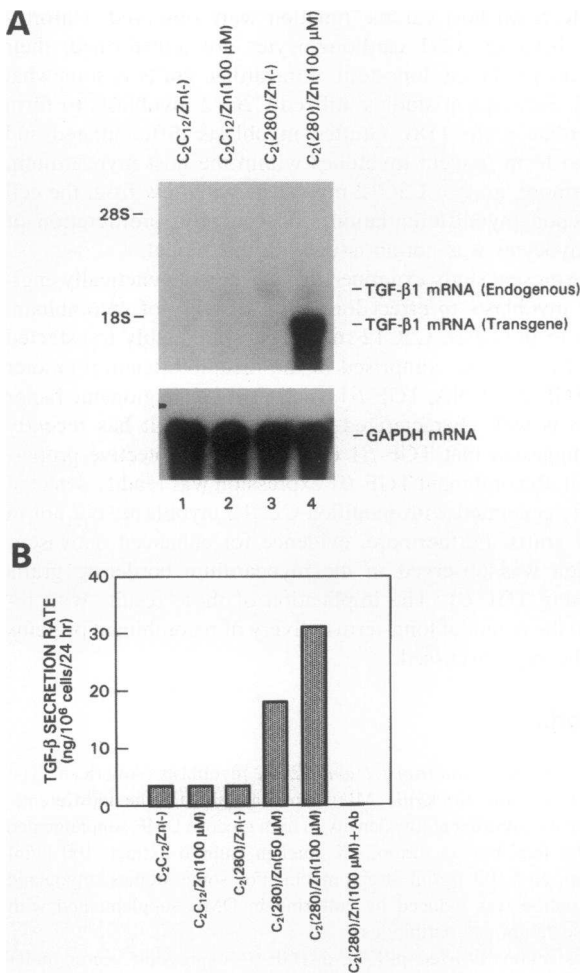

Figure 1. (*A*) Northern blot analysis of TGF-β1 expression. RNA isolated from C2C12 or C2(280) cells grown in the absence or presence of zinc was probed with a recombinant TGF-β1 cDNA. High levels of recombinant TGF-β1 transcripts (1.8 kb) are detected only in C2(280) cells grown in the presence of zinc sulfate. Low levels of endogenous TGF-β1 transcripts (2.5 kb) were observed in all samples. The blot was reprobed with a GAPDH cDNA to confirm the fidelity of the RNA samples. (*B*) CCL-64 growth inhibition assay. Conditioned medium from C2C12 or C2(280) cells grown in the absence or presence of zinc sulfate was screened for biologically active TGF-β1 using a CCL64 mink cell growth inhibition assay. High levels of constitutively active TGF-β1 were present in C2(280) cells grown in the presence of heavy metal. Preincubation of medium with an inhibitory antibody confirmed that the biological activity was due to TGF-β.

grafts. This tendency is documented by the selected photomicrographs in Fig. 2, *E* and *F*, which depict the extremes of capillary density observed in the myocardium bordering C2(280) and C2C12 grafts, respectively.

The vWf immunohistological results suggested the possibility of enhanced myocardial angiogenesis in the TGF-β1 expressing animals. However, given that the heart is highly vascularized to begin with, it was difficult to objectively ascertain the extent of neovascularization using an assay measuring blood vessel density. An assay able to monitor blood vessel growth would provide a more useful assessment of the in vivo effects of targeted TGF-β1 expression. Since blood vessel growth is dependent upon the proliferation of all cellular components of the vessel, we reasoned that assessment of [^{3}H]thymidine incor-

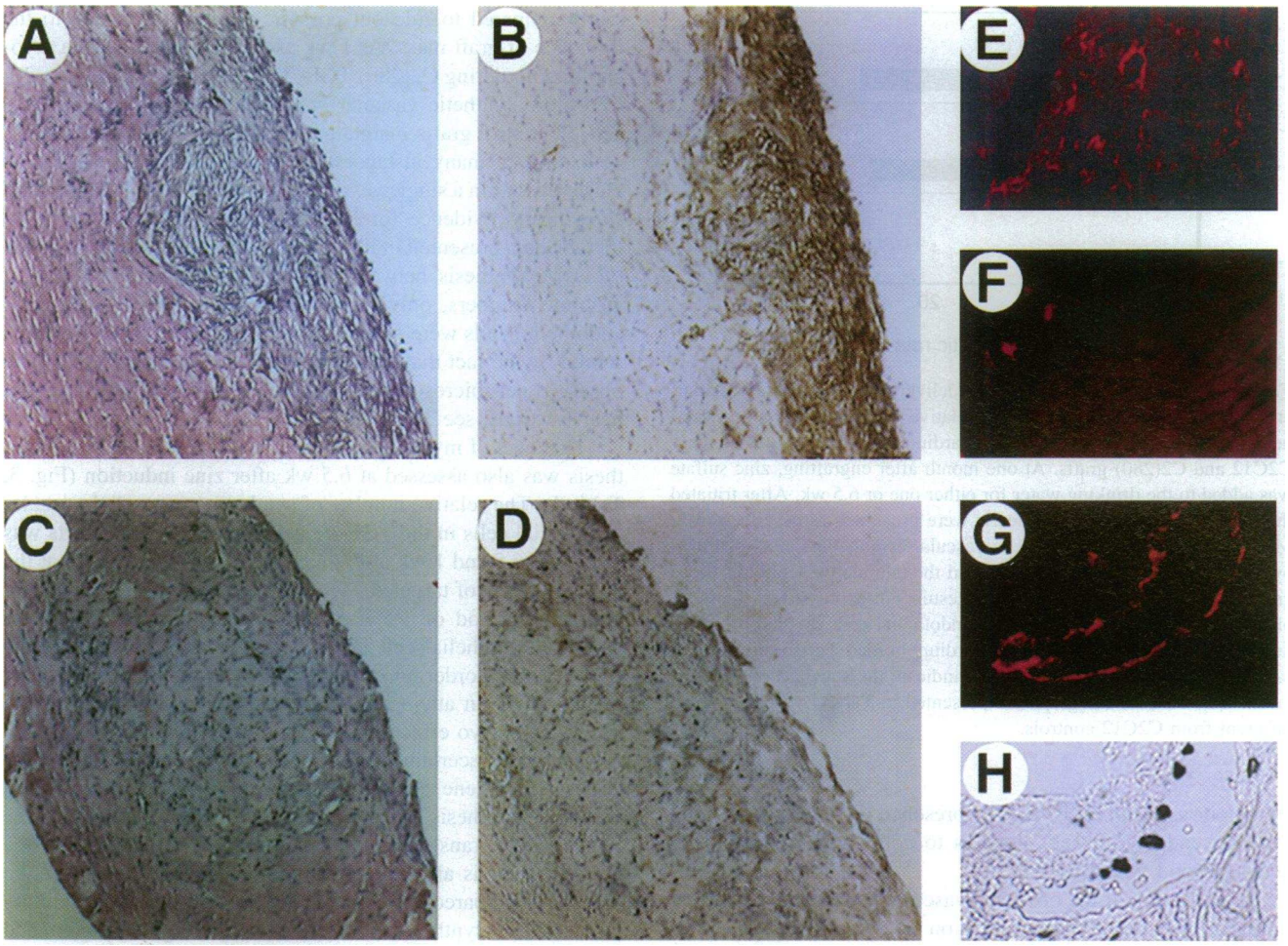

Figure 2. Histologic analysis of C2(280) and C2C12 intracardiac grafts. (*A*) H and E analysis of C2(280) intracardiac graft 6 wk after implantation and 2 wk after zinc sulfate induction. Graft can be distinguished from host myocardium by the weak eosin staining. ×100. (*B*) Immunohistologic analysis for TGF-β1 expression in a C2(280) intracardiac graft (section serial with that depicted in *A*). Brown immunoperoxidase reaction product indicates high levels of recombinant TGF-β1 expression in the engrafted cells. X100. (*C*) H and E analysis of C2C12 intracardiac graft 6 wk after implantation and 2 wk after zinc sulfate induction. Graft can be distinguished from host myocardium by the weak eosin staining. X100. (*D*) Immunohistologic analysis for TGF-β1 expression in a C2C12 intracardiac graft (section adjacent to that depicted in *C*). Absence of brown immunoperoxidase reaction product indicates that the graft is not expressing high levels of TGF-β1. X100. (*E*) Immunohistology for vWf localization in the myocardium bordering a TGF-β1 expressing C2(280) graft. The graft is localized in the upper left corner of the micrograph. Note the high myocardial capillary density as evidenced by vWf immunoreactivity. X400. (*F*) Immunohistology for vWf localization in the myocardium bordering a control C2C12 graft. The graft is localized in the upper left corner of the micrograph. The absence of significant myocardial vWf immunoreactivity suggests relatively low capillary density; positive signal can be seen within the graft itself. X400. (*G*) vWf immunohistology. C2(280) engrafted animals maintained on high dietary zinc for 6.5 wk were given a bolus injection of [^{3}H]thymidine, and heart sections were processed simultaneously for vWf immunohistology and autoradiography. The panel shows an epifluorescence view of a blood vessel in the myocardium adjacent to the TGF-β1 expressing C2(280) graft. X1,000. (*H*) Same field depicted in *G* above, but viewed under bright field microscopy. Note the presence of six synthetic vascular endothelial cells, as evidenced by the presence of nuclear-localized silver grains in the autoradiogram. X1,000.

poration in vascular endothelial cells would provide a nonsubjective, easily scoreable means with which to quantitate the in vivo effects of recombinant protein expression.

Accordingly, mice engrafted with either C2C12 or C2(280) myoblasts were maintained on normal rodent chow without zinc supplement for a period of 1 mo to allow the myoblasts to terminally differentiate (given that there are trace levels of zinc in both the drinking water and food, we have designated this as a low zinc diet). Animals were then placed on a high zinc diet (25 mM zinc sulfate supplement in the drinking water) for a period of either 1 or 6.5 wk. On the final day of the procedure, animals were given a bolus injection of [^{3}H]thymidine, and sacrificed 14 h later. Fig. 2 shows a representative section of the heart from one of the C2(280) engrafted animals. The section was stained with an anti-vWf antibody and then processed for autoradiography. Immunofluorescence photomicroscopy clearly revealed the presence of a large blood vessel in the myocardium bordering the TGF-β1 expressing graft (Fig. 2 *G*). Bright field analysis of the same field indicates that six of the endothelial cells in this blood vessel were actively synthesizing DNA, as evidenced by the presence of nuclear-localized silver grains in the autoradiograph (Fig. 2 *H*). Thymidine incorporation was also evident in the underlying media; however, given the ease with which vascular endothelial cells can be identified in H and E stained sections, assessment of DNA synthesis in these cells was used for quantitation purposes. As indicated

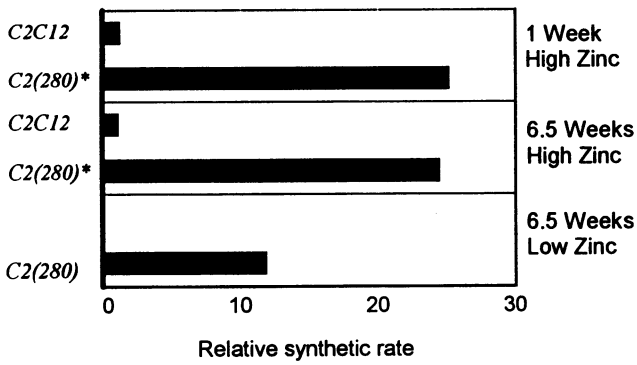

Figure 3. Effects of chronic TGF-β1 delivery on myocardial vascular endothelial cell DNA synthesis. The relative number of synthetic vascular endothelial cells in the graft/myocardium border is compared for C2C12 and C2(280) grafts. At one month after engrafting, zinc sulfate was added to the drinking water for either one or 6.5 wk. After tritiated thymidine administration, the hearts were processed as described in Methods. The number of synthetic vascular endothelial cells (identified by autoradiography) were counted, and the cumulative length of the graft/myocardium border measured. Results are expressed as the ratio of the number of synthetic vascular endothelial cells divided by the length (arbitrary units) of graft/myocardium border. The mean value for each experiment is shown; error bars indicate the standard error of the mean. Raw data for the graph are presented in Table I. *Statistically different from C2C12 controls.

above our assumption is that the presence of DNA synthesis in vascular endothelial cells equates to cell division and consequently blood vessel growth.

To determine if the observed vascular endothelial cell DNA synthesis was due to the expression of recombinant TGF-β1, synthetic activity was compared between hearts containing either C2C12 or C2(280) grafts (Fig. 3, Table I). Vascular endothelial cell DNA synthesis was monitored after either 1 or 6.5 wk of zinc induction. To normalize the results within a given experimental intervention, the number of synthetic endothelial cells was divided by the cumulative length of the graft/myocardium border. As such, the data reflect the average number of synthetic endothelial cells per unit length of the graft perimeter. Analyses were limited to those myocardial vascular endothelial cells which resided within 500 μm of the graft/myocardium border. Only similar sized ($\leq$15% difference) and aged grafts were compared to rule out possible complications due to the influence of graft mass. At 1 wk after zinc induction, the myocardium bordering C2(280) TGF-β1 expressing grafts had ~20-fold more synthetic vascular endothelial cells as compared to C2C12 control grafts maintained on the same high zinc diet ($P < 0.001$). In many instances, the synthetic cells tended to be clustered within a single vessel, with other vessels on the section showing no evidence for DNA synthesis. It should be noted that the data presented in Fig. 3 represent only the relative levels of DNA synthesis between the two graft types. In terms of absolute numbers, only a small fraction of the total vascular endothelial cells were synthetic, an observation which is underscored by the fact that on average only 2.9 synthetic cells were observed per microscopic section (35 synthetic cells/12 whole-heart sections, see Table I).

The level of myocardial vascular endothelial cell DNA synthesis was also assessed at 6.5 wk after zinc induction (Fig. 3, Table I). The relative number of synthetic myocardial vascular endothelial cells in the TGF-β1 expressing C2(280) hearts was similar at 6.5 and 1 wk after zinc induction, suggesting that the in vivo effects of targeted TGF-β1 expression are sustained for at least a period of 1.5 mo. Once again, only low levels of vascular endothelial cell DNA synthesis were detected in the myocardium bordering control C2C12 grafts at 6.5 wk after induction. In an attempt to further support the notion that the observed in vivo effects were due to recombinant TGF-β1 expression, we ascertained whether a dose-response relationship between transgene activity and myocardial vascular endothelial cell DNA synthesis could be detected. As indicated above, the MT-C2(280) transgene is inducible in vitro (Fig. 1), and the MT promoter is also known to be inducible in vivo (29). We therefore compared the extent of myocardial vascular endothelial cell DNA synthesis in mice with C2(280) grafts maintained on either high or low dietary zinc for 6.5 wk (that is, animals with or without dietary zinc supplement; see Fig. 3, Table I). As expected, anti-TGF-β1 immunohistochemical analyses confirmed higher levels of recombinant protein expression in animals with high dietary zinc as compared to those with low zinc (not shown). Intermediate levels of synthetic myocardial vascular endothelial cells were observed in the low zinc vs high zinc animals (11.9±2.4 vs. 24.5±14.5, respectively), suggesting that the observed in vivo effect was enhanced with elevated dietary zinc, and consequently elevated transgene activity. Although a clear tendency towards intermediate levels of vascular

Table I. Effects of Targeted Recombinant TGF-β1 Expression on Vascular Endothelial DNA Synthesis in Myocardium Bordering Intracardiac Grafts

	C2C12 1 wk High Zn	C2(280) 1 wk High Zn	C2C12 6.5 wk High Zn	C2 (280) 6.5 wk High Zn	C2(280) 6.5 wk Low Zn
Sections examined	14	12	24	33	17
Number of grafts	2	3	4	4	3
[^{3}H]Thymidine positive vascular endothelial cells	3	35	2	89	17
Mean length of graft/myocardium border (arbitrary units)	996±133	1119±122	1111±46	1202±121	872±43
Number synthetic cells/unit length border	1.3±0.5‡	25.2±3.8*	1.2±0.5‡	24.5±2.5*	11.9±0.6*

Results are presented as mean±the standard error. * Significantly different from C2C12 control grafts at the same time point, $P < 0.05$. ‡ Controls were not significantly different.

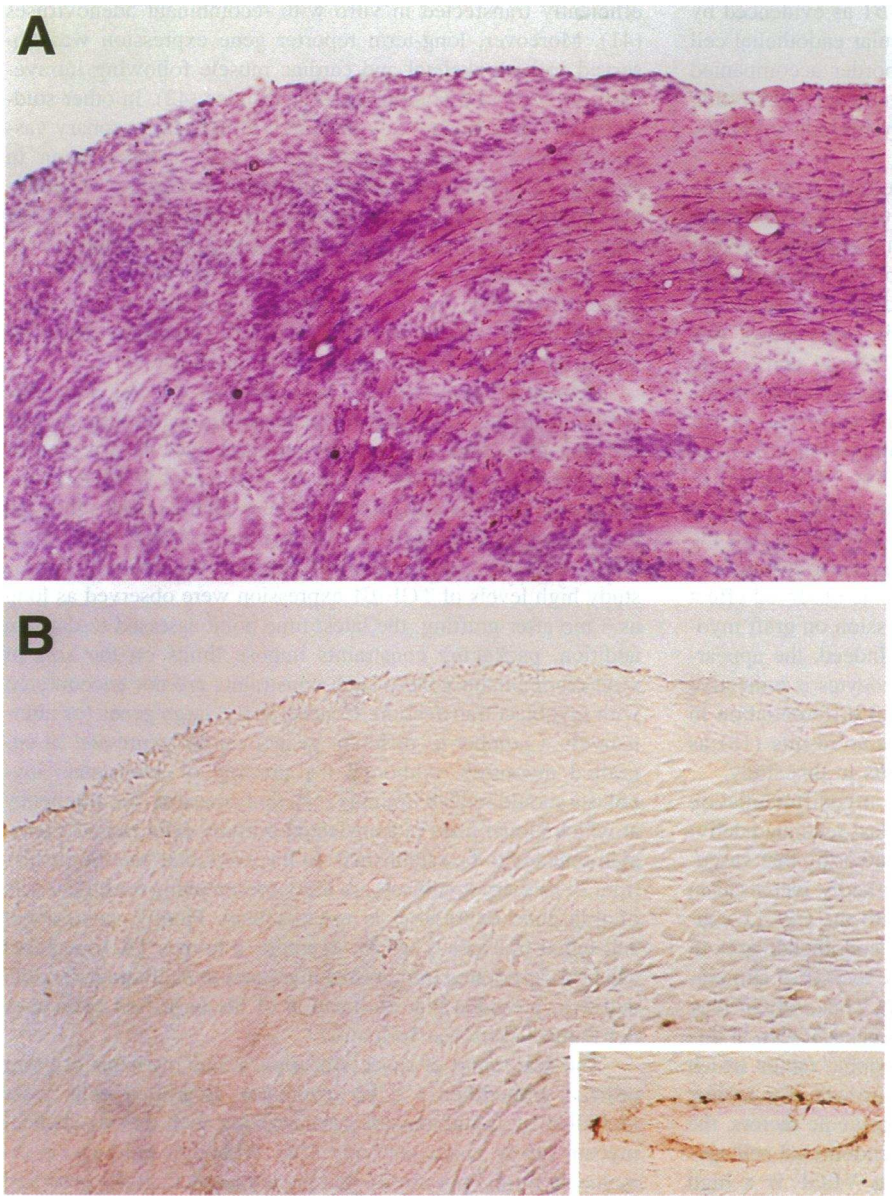

Figure 4. Assessment of C2(280) graft rejection. (*A*) H and E analysis of a C2(280) graft from a mouse maintained on high dietary zinc. Note the presence of nascent graft myotubes and the absence of obvious monocyte infiltration. X400. (*B*) Immunohistologic analysis of the same graft depicted in panel *A* with an anti-leukocyte antibody. Note the absence of infiltrating leukocytes, suggesting the absence of significant immune rejection. X400. The inset shows anti-leukocyte immunohistology of the contents of a large blood vessel from a remote region of the same heart. Note presence of strong leukocyte immunoreactivity, providing a positive control for the assay. X400.

endothelial cell DNA synthesis was observed for the C2(280) engrafted animals maintained on low dietary zinc, the differences did not reach statistical significance with the limited number of animals employed in this study.

The TGF-β1 cDNA used in this study was isolated from a porcine cDNA library (21). Given that this protein is foreign, experiments were initiated to determine if an immunologic response could be detected in C2(280) engrafted hearts. H and E analyses failed to detect obvious monocyte infiltration in TGF-β1 expressing C2(280) grafts (Fig. 4 *A*). This observation was confirmed by immunohistology with antibody specific for the common leukocyte antigen CD25. This antibody recognizes all lymphoid and hematopoietic cells, including B and T lymphocytes, monocytes and granulocytes. No immunoreactivity was observed in the TGF-β1 expressing C2(280) grafts from animals maintained on high dietary zinc for a period of 6.5 wk (Fig. 4 *B*). Strong anti-leukocyte signal was observed in a remote blood vessel, providing a positive control for the experiment. The absence of a detectable immune response further supports the contention that the observed in vivo effects in the C2(280) engrafted hearts is due to the biological activity of TGF-β1, as opposed to an auto-immune phenomenon. Finally, it should be noted that thymidine incorporation also revealed that low levels of C2(280) myocyte DNA synthesis could be detected in animals maintained on both low and high dietary zinc. This observation is consistent with the known inhibitory effect of TGF-β1 on myodifferentiation (30, 31), and most likely accounts for the somewhat undifferentiated appearance of the C2(280) grafts mentioned above. Similar levels of DNA synthesis were not apparent in the control C2C12 grafts which do not express recombinant TGF-β1, in agreement with our previous results (18).

Discussion

This study demonstrates that intracardiac grafts produced with genetically altered C2C12 myoblasts can be used to effect long-term delivery of recombinant proteins to the heart. C2C12 cells transfected with a modified TGF-β1 cDNA constitutively secreted biologically active protein. Intracardiac grafts comprised

of these cells expressed recombinant TGF-β1 as evidenced by immunohistologic analyses. Increased vascular endothelial cell DNA synthesis at the graft-myocardium border accompanied TGF-β1 expression in a dose-dependent manner, suggesting that therapeutic responses could potentially be induced in the myocardium using this approach.

Although this study indicates that cellular grafting approaches can be used for the long-term delivery of recombinant molecules to the heart, the model is not without flaws. Specifically, the subpopulation of C2(280) myoblasts failing to undergo terminal differentiation, as evidenced by tritiated thymidine incorporation, deserves mention. It is well known that exogenous growth factors, including both TGF-β1 and α- and β-fibroblast growth factors (FGFs), can interfere with myogenic differentiation in culture (30–32). A recent study has shown that activated protein kinase C (PKC) mimics the effects of TGF-β1 and FGFs on myodifferentiation (33). This study further demonstrated that FGF stimulation induced PKC-mediated phosphorylation of myogenin, a modification which renders the protein unable to bind to DNA and thus unable to actuate the myodifferentiation program. Consequently, the observed effect of metallothionein/TGF-β1 transgene expression on graft myodifferentiation in vivo was not surprising. Indeed, the appearance of thymidine incorporation in graft myocytes is indicative of their failure to achieve complete terminal differentiation in this model, and is in contrast to our previous results (18) as well as with the nontransfected control grafts in this study.

Although the effects of TGF-β1 on the myodifferentiation of engrafted cells precludes the use of myoblast-mediated delivery in a clinical setting, it is nonetheless noteworthy that vascular endothelial cell DNA synthesis was markedly increased in hearts with C2(280) grafts as compared with the C2C12 controls. Recent studies suggest that vascular endothelial growth factor (VEGF) is rapidly induced in response to TGF-β stimulation (Kari Alitalo, personal communication). Thus, VEGF may mediate the apparent TGF-β1 induced angiogenesis seen in this model. VEGF is an hypoxia-induced angiogenic factor which is thought to play a critical role in tumor growth (for review see reference 34). Unlike other putative angiogenic factors, the mitogenic activities of VEGF appear to be endothelial cell specific (35). It has been recently shown that VEGF is a high affinity ligand for a receptor tyrosine kinase known as flk-1 (for fetal liver kinase-1, see reference 36). In situ analyses show correlations in the spatial and temporal patterns of VEGF and flk-1 expression in developing mouse embryos, lending further support to the notion that VEGF is a major regulator of angiogenesis. Given these considerations, long-term expression of VEGF in intracardiac grafts might induce neovascularization in the adult myocardium without the deleterious effects on myodifferentiation observed with TGF-β1 in the present study.

Several other approaches to effect long-term delivery of recombinant molecules to the myocardium have recently been described. One approach is based on the observation that DNA injected directly into skeletal muscle beds is expressed in vivo (37). Subsequent studies from several groups have shown that this approach is also viable in the heart (38, 39). Although the injected DNA exhibited relatively low transfection efficiencies and a highly variable half-life in the initial studies, reporter gene expression as long as 19 mo after injection into skeletal muscle has recently been reported (40). The injected DNA remained episomal and did not replicate in this study. A second approach being explored for myocardial gene therapy relies on the use of recombinant adenovirus. Cardiomyocytes can be efficiently transfected in vitro with recombinant adenoviruses (41). Moreover, long-term reporter gene expression was observed in both skeletal and cardiac muscle following intravenous delivery of recombinant virus in mice (42). In other studies, adenovirus was readily delivered to both the coronary vasculature and to heart muscle via cardiac catheterization in rabbits (43). These approaches hold great promise for the delivery of gene products to the myocardium, although problems are currently encountered with both temporal limitations of transgene expression and "down-streaming" effects (i.e., viruses delivered to the myocardium can find their way to other organs via the blood stream). This latter problem might be solved by the use of cell type-specific promoters.

Myocardial gene therapy via intracardiac grafting may offer several technical advantages over these alternative approaches. For example, since transgene expression can be assessed in vitro before grafting, and since the transfected DNA is stably integrated, recombinant protein delivery should not be subjected to the temporal limitations currently encountered with direct DNA injections and adenoviral transfections. Indeed, in this study high levels of TGF-β1 expression were observed as long as 3 mo after grafting, the latest time point assessed to date. In addition, packaging constraints impose limits on the size of viral recombinant genes. Such constraints are not encountered with myoblast transfection. Consequently, large genes (or alternatively a number of different genes) can be expressed in engrafted myotubes. Although the grafting of autologous myoblasts should reduce immune rejection concerns, the frequency at which clonal lines of transfected primary cells can be generated remains to be established. In the event that batch transfections of primary myoblasts are used, prescreening for high levels of transgene expression is not practical. Finally, conditional cell-lethal transgenes (as for example, a herpes TK transgene) can be incorporated into grafted myocytes to facilitate reversible delivery, provided that elimination of the engrafted cells does not induce overt scar formation.

The first report of successful intracardiac myoblast grafting utilized unmodified C2C12 myoblasts. Engrafted cells were identified by immunohistological staining with an anti-skeletal myosin heavy chain antibody (18). Although unlikely, it remained a formal possibility that the intracardiac grafts observed in this previous study may have arisen as a consequence of C2C12 myoblast-mediated induction of myogenic differentiation in host cardiac fibroblasts. In contrast, the present study used genetically modified myoblasts. The observed expression of recombinant TGF-β1 confirms that the grafts did not arise via a mechanism relying on the myoconversion of cardiac fibroblasts.

In summary, this study demonstrates that genetically modified skeletal myoblasts can successfully target long-term expression of biologically active recombinant protein when grafted into the myocardium. Long-term expression of recombinant TGF-β1 was accompanied by increased DNA synthesis in vascular endothelial cells, consistent with a sustained angiogenic response. In addition, several other approaches are currently being developed to effect cardiac gene therapy. In now remains to be determined if local, long-term delivery of therapeutic agents via somatic cell gene therapy is superior to traditional pharmacological approaches.

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