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Overexpression of insulin-like growth factor-1 in the heart is coupled with myocyte proliferation in transgenic mice

(DNA synthesis/myocyte number/myocyte volume/cardiomegaly)

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ABSTRACT **Transgenic mice were generated in which the cDNA for the human insulin-like growth factor 1B (IGF-1B) was placed under the control of a rat α-myosin heavy chain promoter. In mice heterozygous for the transgene, IGF-1B mRNA was not detectable in the fetal heart at the end of gestation, was present in modest levels at 1 day after birth, and increased progressively with postnatal maturation, reaching a peak at 75 days. Myocytes isolated from transgenic mice secreted 1.15 ± 0.25 ng of IGF-1 per 10^6 cells per 24 hr versus 0.27 ± 0.10 ng in myocytes from homozygous wild-type littermates. The plasma level of IGF-1 increased 84% in transgenic mice. Heart weight was comparable in wild-type littermates and transgenic mice up to 45 days of age, but a 42%, 45%, 62%, and 51% increase was found at 75, 135, 210, and 300 days, respectively, after birth. At 45, 75, and 210 days, the number of myocytes in the heart was 21%, 31%, and 55% higher, respectively, in transgenic animals. In contrast, myocyte cell volume was comparable in transgenic and control mice at all ages. In conclusion, overexpression of IGF-1 in myocytes leads to cardiomegaly mediated by an increased number of cells in the heart.**

Insulin-like growth factor-1 (IGF-1) belongs to the insulin family of peptides and acts as a growth factor in many tissues and tumors (1). Limited information is available on the effects of IGF-1 on the growth of cardiac myocytes. In neonatal ventricular myocytes in culture, IGF-1 activates DNA synthesis (2, 3) and the expression of myosin light chain-2, troponin, and α-skeletal actin (4), which are consistent with a hyperplastic and hypertrophic response of these cells. However, long-term cultures of adult myocytes react to the addition of IGF-1 by increasing only the formation of myofibrils in the cytoplasm (5). An up-regulation of IGF-1 mRNA in the myocardium occurs in pressure overload hypertrophy *in vivo* (6, 7), and this adaptation has been linked to myocyte hypertrophy. Recent observations have reported that acute ventricular failure is characterized by enhanced expression of IGF-1 and IGF-1 receptor (IGF-1R) in the stressed myocytes, which is followed by DNA replication, nuclear mitotic division, and cell proliferation (8, 9). In line with these findings, the decline in DNA synthesis and cellular hyperplasia with postnatal myocardial development (10) is accompanied by attenuation in the expression of IGF-1 and IGF-1 receptor in myocytes in spite of ongoing cellular hypertrophy (11). However, a cause and effect relationship between IGF-1 and myocyte growth *in vivo* has not been established. For this purpose, a construct was made in which the human IGF-1B cDNA was placed under the control of the rat α-myosin heavy chain (α-MHC) promoter (12), which was then introduced as a transgene in FVB/N mice. This communication presents the effects that this transgene has on cardiac myocytes and on the whole animal, in heterozygous mice, designated as FVB.Igf+/−. Moreover, the consequences of this transgene on the hemodynamic characteristics of the heart were assessed at various phases of postnatal maturation and adult life.

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MATERIALS AND METHODS

Construction of the Transgene. The plasmid pαMHC 6.10 containing the α-MHC promoter (a kind gift of Bernardo Nadal-Ginard, Boston) was digested with *Eco*RI. The fragment containing the vector (pUC19) and the *Eco*RI/*Bam*HI fragment of the α-MHC promoter was religated to create pSMHC plasmid. The IGF-1 cDNA (B form) was excised from a plasmid (a kind gift of Peter Rotwein, Washington University, St. Louis) by *Xho*I and *Eco*RI digestion. This fragment contains IGF-1B cDNA from nucleotide 170 *Rsa*I to nucleotide 1144 *Eco*RI. The cDNA was cloned into KS Bluescript (Stratagene) and digested with *Eco*RI and *Sal*I to create pKSIGF-1B. The plasmid was subsequently digested with *Xho*I and *Sca*I and the fragment containing the IGF-1B cDNA and part of the vector was isolated and ligated to the *Sca*I/*Sal*I fragment of pGem3 (Promega) to obtain pGKIGF-1B. This plasmid was digested with *Xba*I, and the fragment containing the IGF-1B cDNA was isolated and ligated into the *Xba*I site of pSMHC. The proper orientation was confirmed by restriction analysis and the plasmid was called pE1MIB. This plasmid was digested with *Sal*I and *Sca*I and the fragment containing the α-MHC promoter and IGF-1B cDNA was isolated and cloned into the *Xho*I/*Sca*I fragment of COB12 plasmid (13). The resulting plasmid (pOCME1B) contains the *Eco*RI fragment of the α-MHC promoter, exon 1, intron 1, and part of exon 2 of MHC, IGF-1B cDNA, simian virus 40 polyadenylylation signal, and the vector sequence required to grow the plasmid in *Escherichia coli*. The DNA fragment (2817 bp) introduced into the mice was obtained by digestion of the pOCME1B with *Nde*I (Fig. 1). IGF-1B cDNA (974 bp) contains 155 bp that codes for signal peptide, 210 bp coding for 70 amino acids of mature IGF-1, 234 bp coding for E peptide, and 375 bp of 3′ untranslated region.

Production of Transgenic Mice. FVB/N mice (The Jackson Laboratory) were used as embryo donors. Founder transgenic mice were generated by microinjection of the male pronucleus of fertilized mouse eggs with the 2.8-kb α-MHC/IGF-1B construct and the 4.5-kb tyrosinase minigene (selection marker) (14). The two linear transgene constructs were mixed together in 1:1 ratio prior to microinjection. Microinjected eggs were implanted into the oviduct of pseudopregnant female mice and carried to term. Positive founders were subsequently bred to wild-type FVB/N mice. IGF-1B heterozygotes and nontransgenic littermates from the F_1 generation were selected by using PCR of genomic DNA.

Abbreviations: IGF-1, insulin-like growth factor-1; α-MHC, α-myosin heavy chain; SFM, serum-free medium; CM, conditioned medium; OD, optical density.

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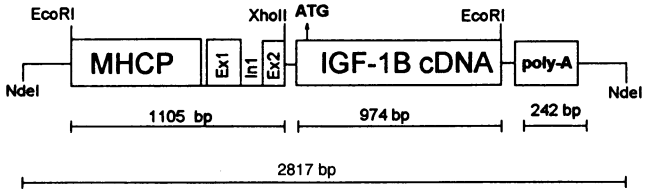

FIG. 1. α-MHC/IGF-1B transgene: *Nde*I fragment (2817 bp) of pOCME1B plasmid was used to produce transgenic mice, which overexpress IGF-1B in the heart. The first open box contains MHC promoter, including 1105 bp of 5′ flanking sequence, exon 1 (Ex1), intron 1 (In1), and part of exon 2 (Ex2) from the rat α-MHC gene (GenBank accession no. K01464). This corresponds to the 5′ untranslated region of the α-MHC gene. The start codon in exon 2 of the α-MHC gene is not present in the construct. The second open box contains a 974-bp fragment of human IGF-1B cDNA (GenBank accession no. M11568). The third open box represents simian virus 40 polyadenylylation signal and the vector sequence required to grow the plasmid in *E. coli*.

Because all IGF-1B positive mice in F_1 generation showed color and all IGF-lB negative littermates were white, it was assumed that the two transgenes integrated into the same chromosomal location. For this reason, further littermates were screened by visual inspection for coat and eye color. Additionally, PCR of genomic DNA was routinely performed to ensure that genetic recombination had not occurred.

Screening of Transgenic Mice by PCR of Genomic DNA. Fragments of tail tissue were cut from 3-week-old mice and incubated overnight at 55°C in 400 μl of lysis buffer (10 mM Tris·HCl, pH 8.3/50 mM KCl/0.45% Nonidet P-40/0.45% Tween 20) and 20 μl of proteinase K (20 mg/ml). Subsequently, samples were incubated at 95°C for 10 min, cooled to room temperature, and spun down to pellet debris. Two microliters of the lysate were used in a total of 30 μl of PCR reaction, performed according to the protocol given by Perkin–Elmer/Cetus. Amplimers and probe for IGF-1B were chosen from the sequence of human cDNA (GenBank accession no. M11568). 5′ sense amplimer, positions 361–380 bp 5′-ATGCTCTTCAGTTCGTGTGT-3′; 3′ antisense amplimer, positions 515–534 bp 5′-CTGACTTGGCAGGCTTGAGG-3′; antisense probe; positions 453–472 bp 5′-AAGCAGCACTCATCCACGAT-3′. The amplification product is 174 bp long. The primers span intron 2 of genomic IGF-1B and should only amplify the transgene.

Ventricular Hemodynamics and Gross Anatomical Parameters. Under chloral hydrate anesthesia (50 mg/kg body weight i.p.), the right carotid artery was cannulated with a fluid filled catheter attached to a P10EZ pressure transducer (Viggo-Spectramed, Oxnard, CA). The catheter was advanced into the left ventricle for the evaluation of left ventricular pressures and the first derivative of pressure (dP/dt) in the closed-chest preparation (8, 9). These functional determinations were performed in mice at 45 days and older. At sacrifice, the weights of the heart and major organs were obtained. Tibial length was measured in mice at 14 days of age and older.

RNA Isolation and Northern Blot Analysis. Total RNA was isolated from heart, skeletal muscle, liver, spleen, lungs, kidney, and ovary of 2.5-month-old IGF-1B transgenic mice and nontransgenic littermates (15). RNA was also extracted from fetal hearts and from hearts at 1, 7, and 14 days and at 1.5, 2.5, 4.5, 7, and 10 months after birth. Radioactive probe was prepared by random priming, using the multiprime DNA labeling system (Amersham) and (≈3000 Ci/mmol; 1 Ci = 37 GBq) [α-^{32}P]dCTP (Amersham). A 1014-bp cDNA probe for human IGF-1B was isolated by *Xba*I digestion of the pOBME1B plasmid. The amount of IGF-1B mRNA was standardized using 18S rRNA content as a control (8, 10, 16).

Isolation of Ventricular Myocytes. Myocytes were isolated by collagenase perfusion according to a procedure repeatedly employed in our laboratory (8–10, 17, 18). Consistent with previous results (8–10, 17, 18), the degree of contamination from nonmyocytes ranged from 1% to 3%. The average yield of myocytes with this method was approximately 2×10^6 cells per heart. One-day-old neonatal myocytes were isolated as described (3, 10).

Radioimmunoassay (RIA). For the determination of IGF-1 plasma levels, 200 μl of blood was obtained, the samples were spun down at 14,000 rpm for 5 min, and the plasma was collected. To prepare conditioned medium (CM), ventricular myocytes were plated on laminin-coated 60-mm dishes in serum-free medium (SFM) and cultured for 48 hr (3). Samples of CM and plasma were spun down at $500 \times g$ for 5 min and acidified with glacial acetic acid (1M). RIA was performed using an IGF-1 RIA kit from Amersham. The radioactivity was measured by an automatic γ-counter at a counting efficiency of 80%. Total and nonspecific binding were determined by performing the RIA without competitor and primary antibody, respectively.

Myocyte Volume. Isolated myocytes were stained by bisbenzimide H33258 (17, 19), and a random sampling of 1000 myocytes in each heart was used to determine the relative frequency of mononucleated, binucleated, and multinucleated cells. Myocyte geometric dimensions were obtained with a computerized image analysis system (Jandel Scientific, Corte Madera, CA). In 1-day-old mice, 200 mononucleated myocytes and 50 binucleated myocytes were measured in each heart. In mice at 45, 75, and 210 days of age, 200 binucleated myocytes and 20 mononucleated, trinucleated, and tetranucleated myocytes from each heart were measured. Isolated cells assume a cross-sectional area that resembles a flattened ellipse. The ratio of the minor axis to the major axis of the ellipse was determined by confocal microscopy (19). Cell volume was calculated assuming an elliptical cross section with a major axis that was equivalent to cell width and a minor axis that was computed from the measured ratios (19). Cell length was measured directly.

Myocyte Number. The total volume of the myocardium was first determined by dividing its weight by the specific gravity of muscle tissue (19). Subsequently, hearts were fixed in 10% formalin and paraffin sections containing the entire cross section of the heart were stained with hematoxylin and eosin for morphometric analysis. Fifty fields were examined at ×1000 with a reticle containing 42 sampling points to determine the volume fraction of myocytes and interstitium in each heart (11, 17, 19). The total volume of myocytes in the heart was then calculated from the product of heart volume and the volume fraction of myocytes. From the volume fraction of myocytes in the myocardium and the proportion of mononucleated, binucleated, trinucleated, and tetranucleated cells, determined in enzymatically dissociated myocytes, the volume percent of each cell population in the tissue was obtained (19). This information, combined with the absolute volume of myocytes in the heart, allowed the estimation of the aggregate volume of mononucleated and multinucleated cells in the myocardium (19). Finally, the numbers of mononucleated, binucleated, trinucleated, and tetranucleated cells in the heart were computed from the quotient of their aggregate volumes and their corresponding average myocyte cell volumes (11, 17, 19).

Incorporation of BrdUrd *in Vitro*. Myocytes obtained from mice at 75 days of age were cultured at a density of 2.0×10^4 cells/cm^2 in SFM (3). The medium was changed 30 min after plating and BrdUrd (10 μM) was added. Cells were fixed 24 hr later. BrdUrd was detected and quantified as described (3). In separate cultures, IGF-1 antibody (20 μg/ml, clone sm 1.2; Upstate Biotechnology, Lake Placid, NY) was added and its effect on DNA synthesis was determined.

Data Collection and Analysis. Autoradiograms were analyzed densitometrically. Optical density (OD) of signals for IGF-1 was divided by the signals for 18S ribosomal RNA and quantitative data were expressed in this manner. All results are presented as mean ± SD. Statistical significance between two measurements was determined with the two-tailed Student's *t* test. Statistical

significance in the multiple comparisons was determined by the Bonferroni method (17).

RESULTS

Production of Transgenic Mice. Two founder animals (male 12 and male 34) selected by screening of 97 mice were used for breeding. By PCR of genomic DNA 10 of 19 mice (F_1 generation from founder 34) were positive for the IGF-1B transgene. These 10 animals were mated with FVB/N mice to obtain an F_2 generation of transgenic heterozygous mice and wild-type littermates. Mice raised from founder 12 did not express the IGF-1B transgene and were not used. IGF-1B positive mice in the F_1 generation showed gray color and IGF-1B negative littermates did not. This characteristic was confirmed in subsequent generations. Difficulty was experienced in breeding transgenic males to transgenic females, and we have therefore carried out the present studies on mice heterozygous for the transgene, which are designated as FVB.*Igf*+/−, in contrast to the wild-type littermates, designated as FVB/N.

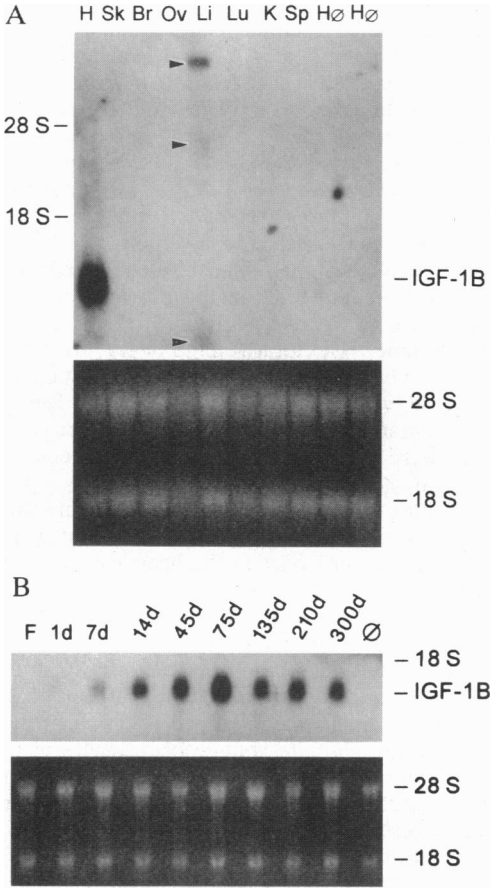

FIG. 2. (*A*) Northern blot detection of IGF-1B mRNA in different tissues of transgenic mice. Each lane contains 20 μg of total RNA isolated from the heart (H), skeletal muscle (SK), brain (Br), ovary (Ov), liver (Li), lungs (Lu), kidneys (K), and spleen (Sp) of a 75-day-old transgenic mouse and from the heart of two wild-type littermates (Hϕ). The blot was hybridized with [α-^{32}P]dCTP-labeled probe isolated by *Xba*I digestion of pOBME1B plasmid. Three splicing forms of endogenous IGF-1 are apparent in the liver (arrowheads). To ensure equivalency of loading, rRNA for each sample is shown in the lower panel. (*B*) Northern blot detection of IGF-1B mRNA in the heart of transgenic mice as a function of age. Each lane contains 20 μg of total RNA isolated from fetal hearts (20 days of gestation) and from hearts at 1, 7, 14, 45, 75, 135, 210, and 300 days after birth. RNA isolated from the heart of 2.5-month-old wild-type littermates (Φ) was used as a negative control. To ensure equivalency of loading, rRNA for each sample is shown in the lower panel.

IGF-1B Expression in FVB.*Igf*+/− Mice. The expression of the IGF-1B transgene was detected by Northern blot analysis (Fig. 2*A*). Total RNA was extracted from different tissues of 75-day-old FVB.*Igf*+/− mice and from hearts of wild-type littermates. The IGF-1B transgene was detected exclusively in the heart of transgenic mice as a 1.0 kb mRNA. No hybridization signal for IGF-1B was detected by both Northern blot and reverse transcription-PCR (30 cycles) in skeletal muscle, brain, ovary, liver, lung, kidney, and spleen. Similarly, IGF-1B mRNA was not observed in the heart of wild-type littermates. Three distinct splicing forms of endogenous IGF-1 were apparent in the liver of both transgenic mice and wild-type as expected (20).

The changes in the expression of IGF-1B in the heart of FVB.*Igf*+/− mice as a function of postnatal maturation and aging are illustrated in Fig. 2*B*. The fetal myocardium failed to reveal the presence of human IGF-1B mRNA by Northern blot analysis. At one day, minimal levels of IGF-1B mRNA were detectable (OD: 0.08 ± 0.02, $n = 3$) and the hybridization signal increased progressively at 7 (OD: 0.24 ± 0.04, $n = 3$), 14 (OD: 0.42 ± 0.05, $n = 3$), and 45 (OD: 0.76 ± 0.07, $n = 3$) days, reaching its peak at 2.5 months after birth (OD: 0.94 ± 0.05, $n = 3$). Levels of human IGF-1B mRNA in the heart remained high up to 10 months of age (OD: 4.5 months: 0.69 ± 0.04, $n = 3$; 7 months: 0.78 ± 0.02, $n = 3$; 10 months: 0.73 ± 0.03, $n = 3$).

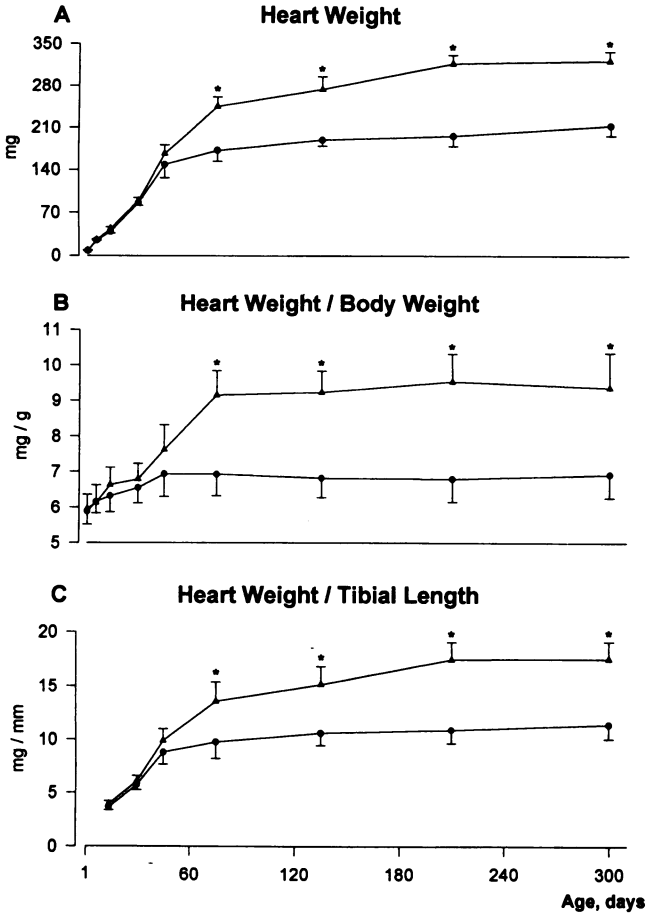

FIG. 3. Changes in the weight of the heart (*A*), heart weight-to-body weight ratio (*B*), and heart weight-to-tibial length ratio (*C*), with postnatal development in transgenic (▲) and wild-type littermates (●) at 1 (transgenic, t : $n = 9$; wild type, wt: $n = 7$), 6 (t: $n = 3$; wt: $n = 3$), 14 (t: $n = 3$; wt: $n = 4$), 30 (t: $n = 3$; wt: $n = 3$), 45 (t: $n = 10$; wt: $n = 8$), 75 (t: $n = 12$; wt: $n = 14$), 135 (t: $n = 15$; wt: $n = 20$), 210 (t: $n = 10$; wt: $n = 15$), and 300 (t: $n = 6$; wt: $n = 6$) days after birth. Results are presented as mean ± SD. *, A value that is statistically significantly different, $P < 0.05$.

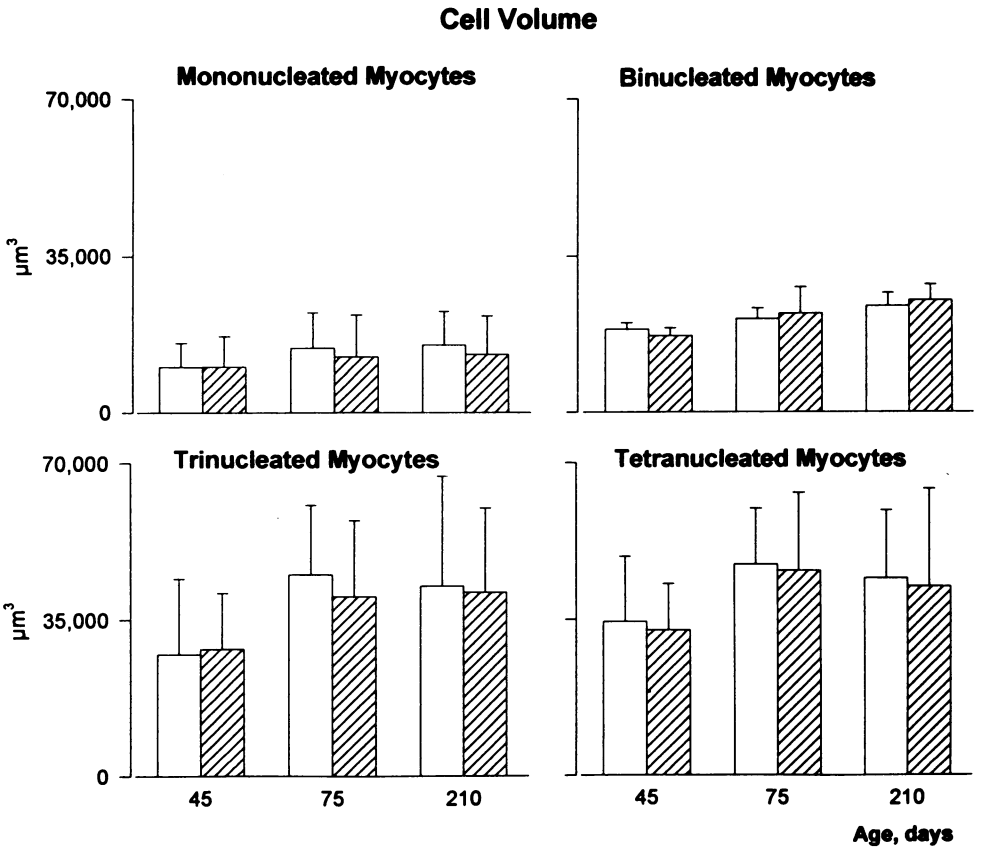

FIG. 4. Effects of postnatal maturation on the volume of the various myocyte classes in the myocardium. Results are presented as means ± SD. Wild type, open bars; transgenic, cross-hatched bars (n = 3 in each group of animals).

IGF-1 Secretion from Myocytes. To determine whether the overexpression of IGF-1B transgene in cardiac muscle cells was accompanied by the secretion of the IGF-1 peptide, IGF-1 was measured in CM collected from primary cultures of ventricular myocytes isolated from 75-day-old transgenic mice and wild-type littermates. This age interval was selected because IGF-1B mRNA levels in the heart peaked at this time (Fig. 2*B*). Myocytes from FVB.*Igf*+/− mice secreted 1.15 ± 0.25 ng of IGF-1 per 10^6 cells per 24 hr (n = 4), whereas myocytes from wild-type littermates secreted 0.27 ± 0.10 ng of IGF-1 (n = 4). The 4.3-fold difference was statistically significant ($P < 0.001$). Since minimal levels of IGF-1B mRNA were noted in the heart of transgenic mice 1 day after birth, ventricular myocytes were isolated from these animals and cultured in SFM for 48 hr. Measurements of IGF-1 peptide in CM showed that similar values were obtained in the two groups of mice (transgenic mice = 0.09 ± 0.05 ng/10^6 myocytes per 24 hr, n = 3; wild-type littermates = 0.085 ± 0.04 ng/10^6 myocytes per 24 hr, n = 3).

Transgenic Mice and Cardiac Characteristic. Systemic arterial blood pressure, left ventricular peak, end-diastolic and developed pressures, left ventricular + and − dP/dt were essentially identical in the two groups of mice up to 300 days of age (data not shown). Body weight was not different in the two groups of animals at birth, and at 6, 14, 30, 45, 75, and 135 days postnatally. However, transgenic mice at 210 (33 ± 3 g, n = 10) and 300 (35 ± 2 g, n = 12) days had a body weight 15% ($P < 0.01$) and 12% ($P < 0.02$) heavier than controls (29 ± 4 g, n = 15; 31 ± 4 g, n = 14). Heart weight was comparable in transgenic and wild-type mice up to 45 days of age, but a 42%, 45%, 62%, and 51% higher value was observed in transgenic mice at 75, 135, 210, and 300 days, respectively (Fig. 3*A*). These differences were statistically significant ($P < 0.001$). When heart weight-to-body weight ratio was considered, a 32%, 35%, 40%, and 35% increase in this parameter was measured in FVB.*Igf*+/− animals at 75, 135, 210, and 300 days, respectively (Fig. 3*B*). These increases were also statistically significant ($P < 0.001$). Similar results were obtained when the ratios of heart weight-to-tibial length were considered (Fig. 3*C*).

Organ Weight and IGF-1 Plasma Levels of FVB.*Igf*+/− Mice. Plasma levels of IGF-1 were measured in mice at 75 days of age. Values of 59 ± 17 ng/ml IGF-1 in transgenics (n = 5) and 32 ± 9 ng/ml IGF-1 in wild-type littermates (n = 5) were obtained. This 84% difference was significant ($P < 0.02$). To establish whether the increased circulating level of IGF-1 in FVB.*Igf*+/− mice affected the size of major organs, the changes in weight of the liver, spleen, kidneys, and brain were analyzed from 14 to 300 days. No significant differences were noted in the weight of these organs up to 30 days. However, at 45 days, the weight of the brain was increased 7% (transgenic: 449 ± 30 mg, n = 10; wild type: 419 ± 20 mg, n = 8; $P < 0.04$) and reached a value of 8% at 300 days (transgenic: 498 ± 17 mg, n = 12; wild type: 461 ± 30 mg, n = 14; $P < 0.04$). Liver weight was increased 11% at 45 days (transgenic: 1298 ± 91 mg, n = 10; wild type: 1168 ± 75 mg, n = 8; $P < 0.01$) and 35% at 300 days (transgenic: 1935 ± 312 mg, n = 12: wild type: 1435 ± 211 mg, n = 14; $P < 0.01$). Similar responses were seen in the spleen and kidneys, but they appeared later, at 135 days, and persisted at 300 days. In contrast, tibial length was comparable in transgenic and wild-type mice from 14 to 300 days.

Transgenic Animals and Myocyte Cell Volume and Number. Immediately after birth, mononucleated myocytes constituted the majority of cells in both groups of animals. However, at 45, 75, and 210 days of age binucleated myocytes represented the prevailing cell type, whereas mononucleated cells were the second most frequent class of cells. Trinucleated and tetranucleated cells comprised significantly smaller fractions of myocytes. Comparisons between transgenic mice and wild-type littermates at each of the four time intervals examined demonstrated that IGF-1 overexpression had no effect on the proportion of the different myocyte populations in the heart (data not shown). The length and cross-sectional area of mononucleated, binucleated, trinucleated, and tetranucleated myocytes were not statistically different between transgenic mice and wild-type littermates at

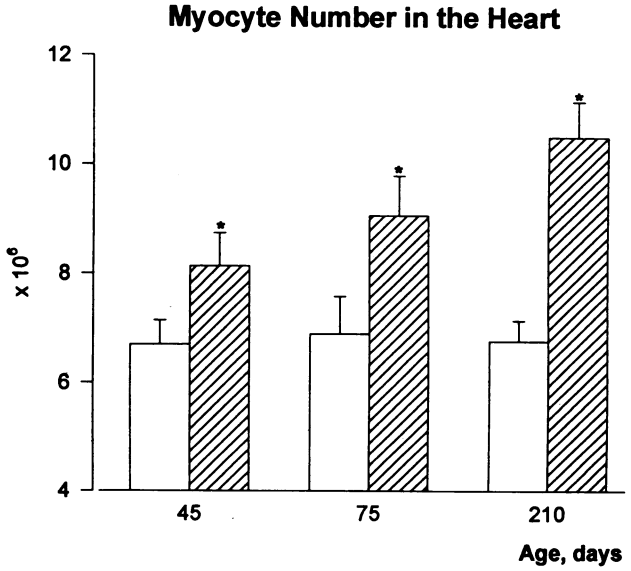

FIG. 5. Effects of postnatal maturation on the aggregate number of myocytes in the heart of wild-type (open bars) and transgenic (cross-hatched bars) mice. Results are presented as mean ± SD. *, A value that is statistically significantly different, $P < 0.05$ ($n = 3$ in each group of animals).

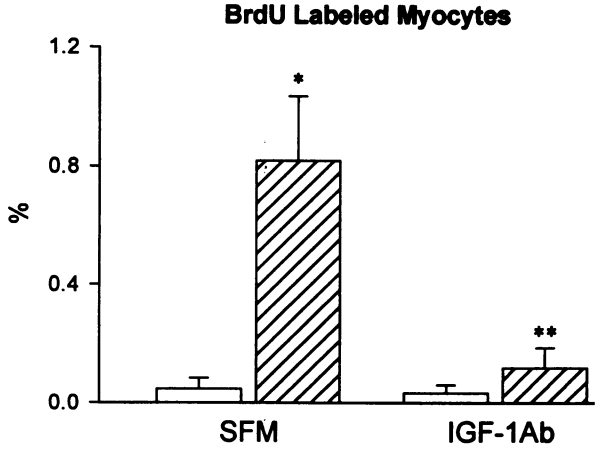

FIG. 6. BrdUrd labeling in myocytes isolated from transgenic (cross-hatched bars) and wild-type (open bars) mice at 75 days of age. SFM, serum-free medium; IGF-1Ab, IGF-1 antibody. Results are presented as means ± SD. *, A value that is statistically different from wild type, $P < 0.05$. **, A value that is statistically different from the corresponding value in SFM.

each of the four age periods studied. As a consequence, myocyte cell volume was comparable at each interval in the two animal groups. At 1 day after birth, only mononucleated and binucleated myocytes were detected; their respective volumes were 1492 ± 208 μm^3 and 1926 ± 595 μm^3 in wild-type mice ($n = 3$), and 1523 ± 187 μm^3 and 1791 ± 878 μm^3 in transgenic animals ($n = 3$). Moreover, the overexpression of IGF-1 in myocytes did not affect the size of these cells at 45, 75, and 210 days of age (Fig. 4).

At 1 day after birth, the volume fraction of myocytes and interstitium in the heart of transgenic mice was 89.9 ± 2.5% and 10.1 ± 2.5%. The proportion of these two constituents was essentially identical in control animals and did not vary with age in the two groups of mice (data not shown). The measurement of the aggregate volume of each class of myocytes in the myocardium in combination with the average volume of mononucleated, binucleated, trinucleated, and tetranucleated cells allowed the computation of the total number of each cell class in the heart. In view of the multiple levels of morphometric analysis employed here, these final measurements were performed in small groups of animals at each time point, which constitutes a limitation to be considered in the interpretation of the collected results. At 1 day there were 4.6 ± 0.2 × 10^6 mononucleated and 78 ± 9.3 × 10^3 binucleated myocytes in wild-type mice, and 4.4 ± 0.3 × 10^6 mononucleated and 104 ± 11 × 10^3 binucleated myocytes in transgenic animals. These differences were not statistically significant. However, IGF-1 overexpression in myocytes resulted in a progressive increase in the number of cells in the heart of transgenic mice at 45, 75, and 210 days of age. This phenomenon resulted in a 21% ($P < 0.005$), 31% ($P < 0.04$), and 55% ($P < 0.002$) increase in the aggregate number of myocytes in the heart of transgenic mice at 45, 75, and 210 days, respectively (Fig. 5).

DNA Synthesis in Ventricular Myocytes *in Vitro*. To determine whether the increase in myocyte number in the heart of transgenic mice was dependent on IGF-1 overexpression, or was influenced by the site of integration of the transgene in the genome, ventricular myocytes from animals at 75 days of age were cultured in serum-free medium for 48 hr. Subsequently, the percentage of cells labeled by BrdUrd was measured (Fig. 6). An average of 0.8% of the myocytes from transgenic mice were found to synthesize DNA, whereas only 0.05% of cells from wild-type mice were stained by BrdUrd. This 16-fold difference was statistically significant ($P < 0.0001$). The addition of IGF-1 antibody markedly attenuated the magnitude of DNA replication in myocytes of transgenic mice. The 85% decrease in this parameter was also statistically significant ($P < 0.0001$).

DISCUSSION

IGF-1 Expression in Cardiac Myocytes. The purpose of the present experiments was to generate transgenic mice, in which the expression of IGF-1 was restricted to the heart. This was achieved by placing the human IGF-1B cDNA under the control of a rat α-MHC promoter (12). The α- and β-MHC genes are expressed exclusively in the heart (21, 22). In embryonic and fetal development, β-MHC predominates in cardiac myocytes of rodents (23), whereas the synthesis of α-MHC is enhanced after birth (24, 25) and, in the young adult animal, α-MHC becomes the main isomyosin present in myocytes (23, 26). This pattern of expression has been confirmed by our findings in which Northern blot analysis did not detect IGF-1 overexpression in the fetal heart although a weak signal was observed at 1 day after birth. The moderately enhanced IGF-1 mRNA level in 1-day-old myocytes was not accompanied by a corresponding increase in the ability of these cells to secrete IGF-1. Thus, the utilization of the α-MHC promoter not only allowed the induction of a cardiac-specific overexpression of IGF-1B, but also restricted this phenomenon to postnatal life. A similar approach has been employed previously to generate mice overexpressing $G_{s\alpha}$ or chloramphenicol acetyltransferase (27, 28).

The overexpression of the IGF-1B transgene in the heart has been confirmed by determining the level of IGF-1 secretion by isolated cardiac myocytes. The measured amount, 1.15 ng of IGF-1 per 10^6 cells, was sufficient to increase by 84% the plasma level of IGF-1. This may explain the secondary effects noted in heterozygous transgenics, which included an increase in the size of several organs, especially the brain and the kidney. This amount of steady-state IGF-1 secretion is substantial, since, although a direct comparison is difficult, a single dose of 5 ng/ml of IGF-1 stimulates DNA synthesis and mitosis of cells in culture (29). Mathews *et al.* (30) have reported a transgenic mouse in which IGF-1, under the control of a metallothionein promoter, was ubiquitously expressed. In these animals, also heterozygous for the transgene, IGF-1 mRNA was overexpressed in several tissues, and yet, the IGF-1 plasma level was increased only 50–60% over wild-type animals, and body weight was increased nearly 75% (30). Organ comparisons are made irrelevant by the fact that the metallothionein promoter was most active in specific tissues like the pancreas, intestine, kidney,and liver, whereas it was not expressed, or was present at very low levels, in the brain

and the heart (30). The similarity in the systemic effects of these two types of transgenic mice suggests that the α-MHC promoter is a very strong promoter in postnatal life, as already indicated by the results of Gaudin *et al.* (28).

Effects of IGF-1 on Myocyte Number and Volume. Our findings show that IGF-1B overexpression in myocytes resulted in an increase in the number of ventricular muscle cells in transgenic mice at 45 days of age. This increase became more apparent at 75 and 210 days. The aggregate number of myocytes in the heart increased by 21% at the earlier interval, whereas a 31% and 55% increase was noted at the subsequent two age periods examined. This increase in cell number affected mostly binucleated myocytes. In contrast, the enhanced formation of IGF-1 had no influence on myocyte cell volume, which was comparable in transgenic and nontransgenic mice at all intervals. The increase in myocyte number detected at 45 days indicates that cell proliferation occurred after birth, but leaves unanswered the question of the timing of this phenomenon. This interval was selected for quantitative analysis because it corresponds to young adult animals and the size of the heart allowed myocyte isolation by collagenase perfusion. This technical difficulty interfered with the characterization of IGF-1 overexpression and myocyte number in the early postnatal period. The increase in myocyte cell number in the heart of transgenic mice could have been influenced by the site of integration of the transgene in the genome. Although such a condition would be expected to result in an increased number of cells at birth, and not to parallel the preferential synthesis of α-MHC postnatally, this potential source of artifact could not be excluded. On the other hand, nearly 1% of myocytes from adult transgenic mice cultured in SFM were shown to undergo DNA replication and this phenomenon was abolished by the addition of IGF-1 antibody. Moreover, a position effect due to the site of integration was excluded by the inability to detect by 30 cycles of RT-PCR IGF-1B mRNA in all organs examined with the exception of the heart. These observations strongly suggest that the overexpression of IGF-1B in myocytes and its secretion were responsible for the proliferative response of these cells in the heart. The approach used here to exclude an integration site bias had the advantage of restricting the morphometric analysis of the myocardium to two groups of animals. However, additional lines of transgenic mice may be developed in the future and an identical quantitation of the heart performed to resolve this potential limitation in our study.

Data in this investigation indicate that the increases in the number of myocytes and cardiac weight with IGF-1 overexpressing were characterized by hemodynamic parameters of ventricular function similar to those measured in nontransgenic mice. This phenomenon implies that the values of systolic and diastolic stress at the cellular level were significantly lower in the larger heart with a greater number of myocytes. Such a condition may potentiate the ability of the myocardium to sustain increases in pressure and/or volume loads as well as the consequences of a diffuse or segmental loss of myocytes.

Although IGF-1 stimulation may induce myocyte hypertrophy *in vitro* (4, 5), the observations summarized above indicate that overexpression of IGF-1 may stimulate an increase in cell number *in vivo*. This selective growth-promoting effect of IGF-1 has been demonstrated previously in neonatal cardiac myocytes in culture (3) and is consonant with the parallel decline in the expression of IGF-1 and DNA synthesis in myocytes during postnatal cardiac development (10). IGF-1 overexpression increased the extent of cell proliferation in the heart and this adaptation was consistent with the mitogenic effect of this growth factor in several cell types (31). Surprisingly, myocyte cellular hypertrophy was not enhanced under this setting. This is at variance with the known action of IGF-1 on the size of other nonmyocytic cells during the cell cycle (32). Perhaps, in our system, myocytes entering the cell cycle may constitute only a small fraction of the entire cell population and the increase in cell size during S and G_2 may be below the level of sensitivity of morphometric methods. However, the continuous increase in cell number that we observed in transgenic mice clearly indicates that IGF-1 sustains cell division in cardiac myocytes, at least up to 7 months of age. The availability of these transgenic mice will allow direct examination of how pathologic interventions affect hearts with various numbers of myocytes, and how myocyte replication may interfere with the initial alterations in cardiac performance and the chronic restoration of normal ventricular hemodynamics.

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