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Long-term expression of isomyosins and myoendocrine functions in ectopic grafts of atrial tissue

(nude mouse/myosin heavy chains/monoclonal antibodies/cardiodilatin/immunogold electron microscopy)

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ABSTRACT **Tissue fragments of newborn rat atria were transplanted under the dorsal skin or into the bed of the anterior tibial muscle of nude mice. After 5–11 weeks, the grafts, which had reorganized into beating atrium-like structures, were analyzed and compared to ventricular tissue transplanted the same way. As revealed by monoclonal antibodies against α- and β-type myosin heavy chains, atrial grafts retained a typical pattern of myosin expression distinct from that of ventricular grafts. The majority of ectopic atrial myocytes contained specific atrial granules in which cardiodilatin-immunoreactive material has been localized. Specific granules and cardiodilatin immunoreactivity were not found in myocytes of ventricular grafts. We conclude that the long-term maintenance of isomyosin expression and of the myoendocrine function of atrial tissue is largely independent of the anatomical environment.**

Tissue culture and transplantation experiments have demonstrated that cardiac muscle is determined early in vertebrate embryogenesis and that it is remarkably autonomous in achieving and maintaining a functional differentiated state (cf. refs. 1, 2). Mammalian atria and ventricles grafted separately to subcutaneous sites of the host displayed different average frequencies of autonomous pulsation, indicating that physiological features characteristic for their site of origin could be retained for several months (3). On the other hand, plasticity of the mammalian heart with respect to the type of myosin expressed has been demonstrated during development and under a variety of pathological conditions (4–15).

The heavy chains of atrial and ventricular myosins belong to either an α-type or a β-type (16). In the rat, the β-type chain is nearly absent from the atria of the newborn animal but is the dominant form of the ventricle, from which, however, it disappears around postnatal day 30. Subsequently a fraction of the ventricular myocytes returns to the synthesis of β-type chains (6, 14). Environmental factors like cardiac mechanical overload and hypothyroidism can drastically shift the relative regional expression of α- and β-type myosin heavy chain genes (17).

We have found previously that minced rat ventricular tissue transplanted into the bed of the anterior tibial muscle of a nude mouse host competed successfully with regenerating host skeletal muscle fibers, maintaining a stable territory in which it organized into a beating structure (18). In the present investigation, we performed transplantation experiments on atrial tissue, the myocytes of which are distinguished from ventricular myocardium by characteristic myofibrillar isoproteins and by their function as endocrine cells (19–22; for a review, see ref. 23). We show that minced atrial tissue reorganized itself at ectopic sites and expressed an isomyosin pattern and myoendocrine functions that distinguish atrial from ventricular grafts. The experimental system of ectopically transplanted tissue can be used to study several aspects of cardiac physiology, especially those related to the production of cardiac hormones and response to external hormones.

MATERIALS AND METHODS

Animals and Surgery. Cardiac tissue was obtained from newborn (1–2 days postnatal) Sprague–Dawley rats; recipient animals were 6- to 10-week-old nude mice (*nu/nu*, strain NMRI). Left and right atria from several hearts were pooled in Earle's balanced salt solution containing 1 g of glucose per liter. Five to six atria were used for one transplantation site after being cut into about 10 pieces (about 100 μm × 120 μm) each. A similar mass of ventricular tissue (usually from the central belt of one heart) was grafted for comparison (18). With "grafts to the muscle bed," one recipient was used per sample. The anterior tibial muscle of the host was removed under pentobarbital anaesthesia, its bed was filled with minced cardiac muscle, and the skin was sutured. With "subcutaneous grafts," a single mouse received two to three tissue samples under the dorsal skin. The experiments have been carried out according to the German laws for the protection of animals with an approved permit.

Monoclonal Antibodies (mAbs). mAbs directed against myosin heavy chains were of the IgG type. mAb F36-2B4 (anti-β-type myosin heavy chain) was selected from a mAb population obtained from a mouse injected with adult human ventricular β-type myosin; it specifically reacted with the rat ventricular β-type myosin heavy chains (12). mAb F88-12F8 (anti-α-type myosin heavy chain) was selected from a mAb population obtained from a mouse injected with an α-like atrial myosin purified from a human left atrium on an immunoaffinity chromatography column by using an anti-β-type myosin mAb (13); mAb F88-12F8 specifically reacted with rat α-type myosin heavy chain. The details of the isolation and characterization of the anti-myosin heavy chain mAbs have been described (13, 24).

Histochemistry and Electronmicroscopy. Enzyme histochemistry for succinic dehydrogenase (EC 1.3.99.1) and immunohistochemistry with commercial horseradish peroxidase (EC 1.11.1.7)-conjugated rabbit anti-mouse IgG (Miles Yeda) were performed on 8-μm frozen sections. First antibodies were either cell culture supernatants diluted with an equal volume of Ca^{2+}/Mg^{2+}-free phosphate-buffered saline

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Abbreviations: CDD, cardiodilatin; mAb, monoclonal antibody; p.o., postoperative (postoperatively); m., musculus.

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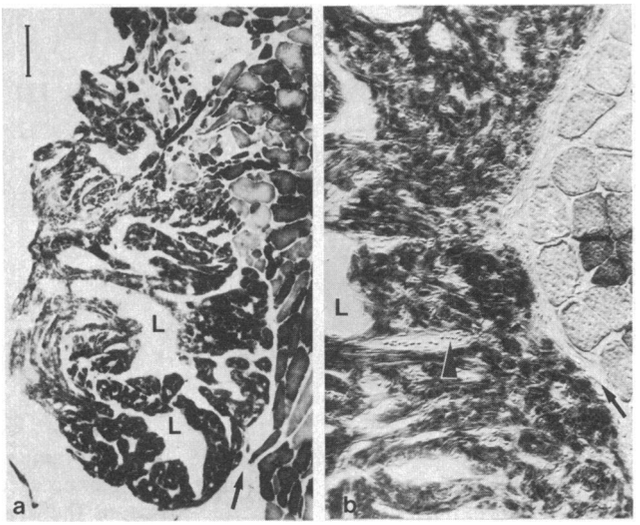

FIG. 1. Reorganization of minced atrial tissue in the skeletal muscle bed. Succinic dehydrogenase staining of frozen sections. Cardiac, skeletal red muscle, and skeletal white muscle show very high, high, and low succinic dehydrogenase activities, respectively. Connective tissue and fat are succinic dehydrogenase negative. (*a*) Atrial transplant 77 days p.o., surrounded by fat and connective tissue (left, not visible), and regenerated host m. tibialis anterior (right). Note the lumen "L" (blood-filled in the living state) and the folded walls typical for atrial morphology. (*b*) Enlarged detail of another transplant (55 days p.o.), showing the narrow connective tissue lining along the border between cardiac and regenerated skeletal muscle (arrow), the lumen (L), and erythrocytes within it (arrowhead). (Interference contrast; bar = 200 μm in *a* and 35 μm in *b*.)

(anti-α-type myosin heavy chain) or ammonium sulfate-precipitated antibodies dissolved in Ca^{2+}/Mg^{2+}-free phos-

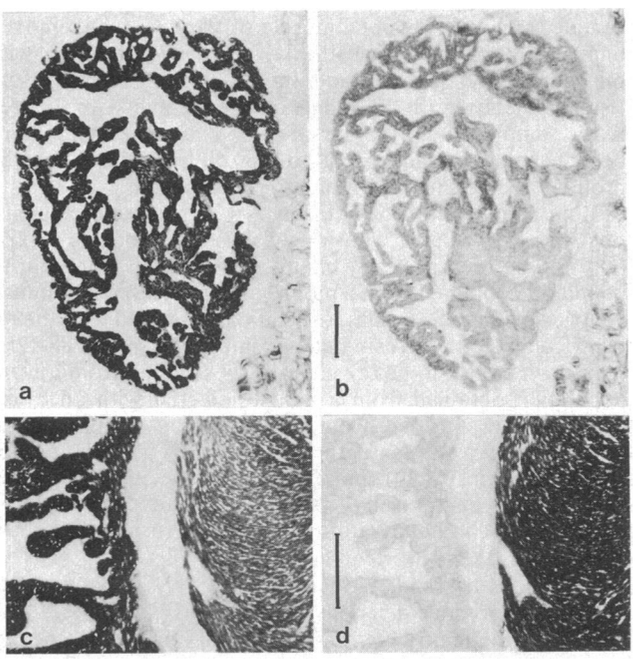

FIG. 2. Atrial transplant and control heart stained with anti-α-type (*a* and *c*) and anti-β-type (*b* and *d*) myosin heavy chain mAbs, respectively. (*a* and *b*) Atrial transplant to the s.c. site analyzed 23 days p.o. Note the morphological organization into a single atrium-like unit. Skin surface is to the left and skin muscle is to the right of the transplant. (*c* and *d*) Control neonatal rat heart, with the border between the atrium (to the left) and ventricle (to the right). (Bars = 200 μm.)

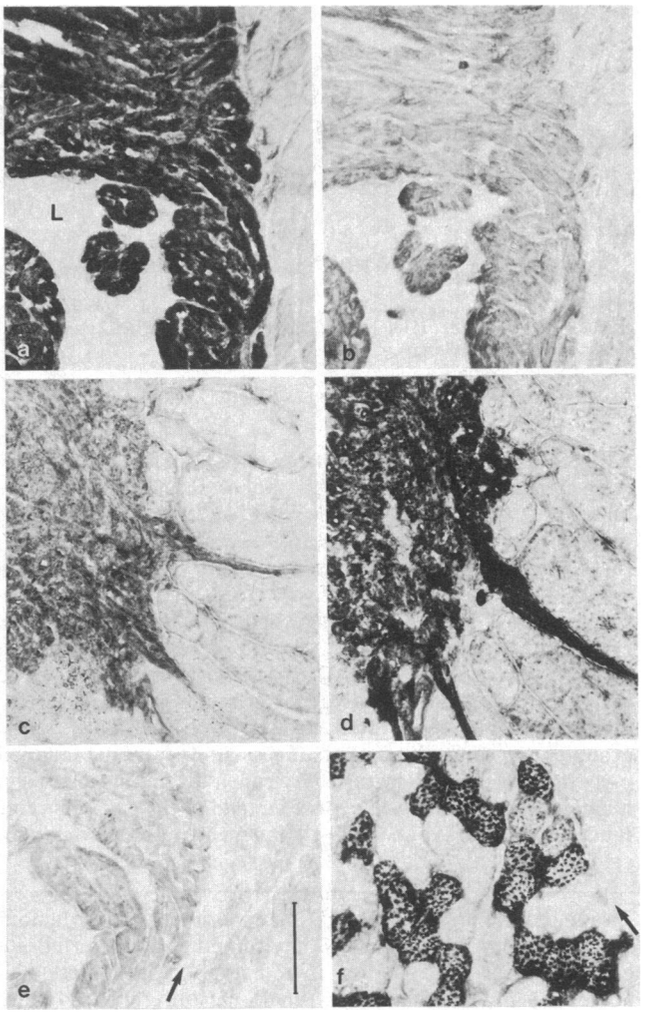

FIG. 3. (*a–e*) Anti-α-type (*a* and *c*) and anti-β-type (*b* and *d*) myosin heavy chain mAb staining of atrial (*a*, *b*, and *e*) and ventricular (*c* and *d*) tissue transplanted to the muscle bed and analyzed on p.o. day 77. "L," lumen. *e* is the negative control, a section from the same block as *a* and *b* with the first antibody omitted. In *a–e*, grafts are to the left and regenerated skeletal muscle (m. tibialis anterior) is to the right (arrow, borderline between the two). (*f*) Skeletal muscle (m. soleus) stained with anti-β-type myosin heavy chain mAb. Note the positive type I (slow) and negative type IIa (fast) fibers. Part of the m. gastrocnemius lacking type I fibers is seen in the upper right (arrow, borderline between the two muscles). (Bar = 100 μm.)

phate-buffered saline (partially purified IgGs, anti-β-type myosin heavy chain). Electron microscopy was done on glutaraldehyde/formaldehyde perfusion or immersion-fixed samples (18).

Cardiodilatin (CDD) immunoreactivity was demonstrated by using a polyclonal rabbit antibody, Ab-HP IV, directed against amino acid residues 117–126 of human or porcine CDD (25). For light microscopic observations, the peroxidase-antiperoxidase method was used (25). The subcellular localization of CDD by immunogold electron microscopy was performed on perfusion-fixed specimens as described (26). The primary label was anti-CDD antiserum applied at a dilution of 1:4000 or 1:8000, the secondary label was a protein A–gold complex (6.2 g/ml; 16-nm colloidal gold).

RESULTS

General Observations. At postoperative (p.o.) days 13–14, atrial transplants could be seen pulsating in the bed of the anterior tibial muscle or at the dorsal subcutaneous site,

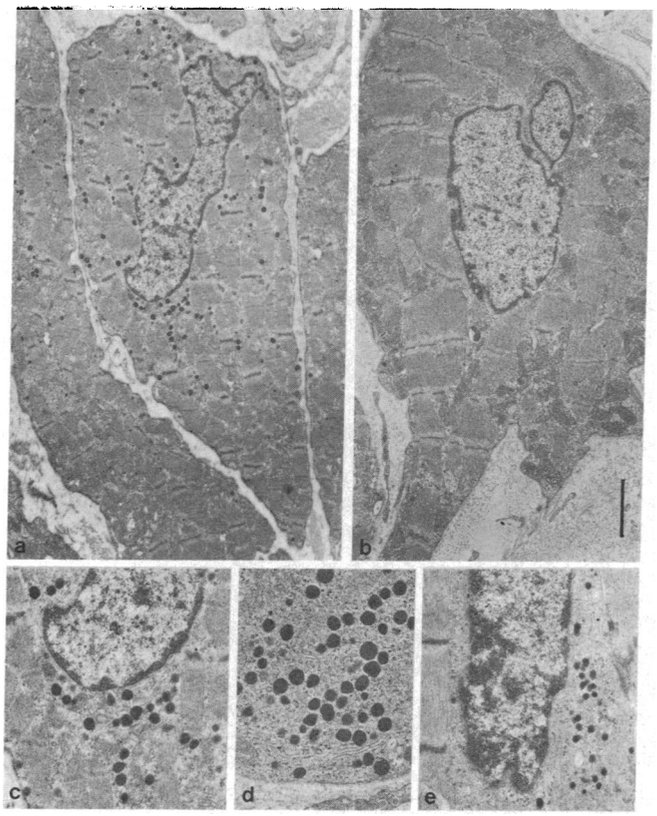

FIG. 4. Ultrastructure of atrial and ventricular grafts. Grafts to the muscle bed are shown. (*a*, *c*, and *d*) Atrial grafts, 37 days p.o. (*b*) Ventricular graft, 38 days p.o. (*e*) Control neonatal rat atrium, showing granules. Note myofibrils in grafts *a* and *b*, but atrial granules only in *a*. *c* and *d* are enlarged areas with atrial granules close to the nucleus (*c*) or in the cell periphery (*d*). (Bar = 2 μm for *a* and *b*; 1 μm for *c*–*e*.)

whereas pulsations of ventricular transplants were only seen after deflecting the skin. Beating frequencies varied between 0.3 and 11 Hz, the highest values being typical for atrial grafts.

Table 1. Isomyosin expression and atrial granules in ectopic myocardial grafts

Origin of tissue	Site	Time p.o., days	Staining		Myocytes with granules†
			αMHC*	βMHC*	
A	Control‡		+++	0	0.97 (354)
V	Control‡		++	+++	0.00 (485)
A	s.c.	23	++, +++	(+)	ND
V	s.c.	23	+++	++	ND
A	m.b.	37	++, +++	(+), +	0.97 (920)
V	m.b.	37	ND	ND	0.006 (641)
A	m.b.	38	+++	(+)	0.67 (438)
V	m.b.	38	+	++	0.02 (146)
A	s.c.	38	ND	ND	0.86 (87)
V	s.c.	38	ND	ND	<0.04 (345)
A	m.b.	77	++, +++	(+)	ND
V	m.b.	77	+	++	ND

Five of 11 independent transplantation experiments are shown. Usually two or three parallel transplantations of each kind were performed per experiment. A, atrium, V, ventricle; p.o., postoperative; MHC, myosin heavy chain; m.b., muscle bed (tibialis anterior); ND, not done.

*The intensity of the horseradish peroxidase/chloronaphthol-staining has been estimated: 0, yellowish background; (+), pale violet on yellowish background (this intensity was frequently seen in negative controls without the first antibody); +, violet; ++, dark violet; and +++, blackish violet.

†The proportion of all myocytes with granules is given, with the total number of myocytes scored in brackets. Myocytes were identified by the presence of myofibrils. Some of the granules seen in ventricular transplants were of doubtful identity, hence proportions given are maximum values.

‡Neonatal control tissue (not transplanted).

Morphology and Histology. The overall structure and the tissue composition of the transplantation sites were revealed by frozen sections stained for succinic dehydrogenase activity (Fig. 1).

As indicated by the beating pattern, both atrial and ventricular (18) tissue fragments were able to reorganize into large morphological and physiological units. The tendency of randomly oriented fragments to form an ordered organ-like unit was particularly striking for the atrial transplants, both

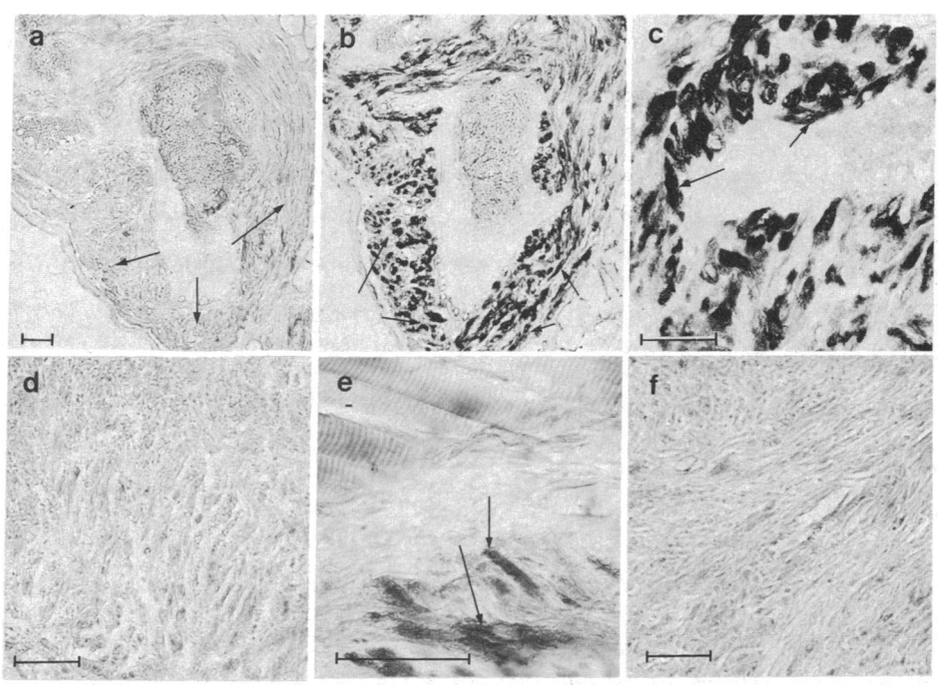

FIG. 5. CDD-like immunoreactivity (CDD-IR) in myocardial grafts. (*a*–*c*) Atrial transplant under the dorsal skin 57 days p.o. The negative control (*a*) is anti-CDD preabsorbed with 1 μg of CDD-99-126 per 20 μl of serum. Arrows point to the myocardium. In *b* is a parallel section to *a*, stained with anti-CDD. Arrows point to strong CDD-IR in myocytes. In *c*, an enlargement from *b* shows the cellular localization of CDD-IR. (*d*) Atrial graft to the muscle bed stained 7 days p.o. with anti-CDD, showing no significant CDD-IR. (*e*) Atrial graft to the muscle bed 57 days p.o., showing the border between regenerated skeletal muscle (unstained, upper half) and atrial muscle (strong CDD-IR, arrows). (*f*) Ventricular graft to the muscle bed stained 57 days p.o. with anti-CDD, showing no significant CDD-IR. (Bars = 50 μm.)

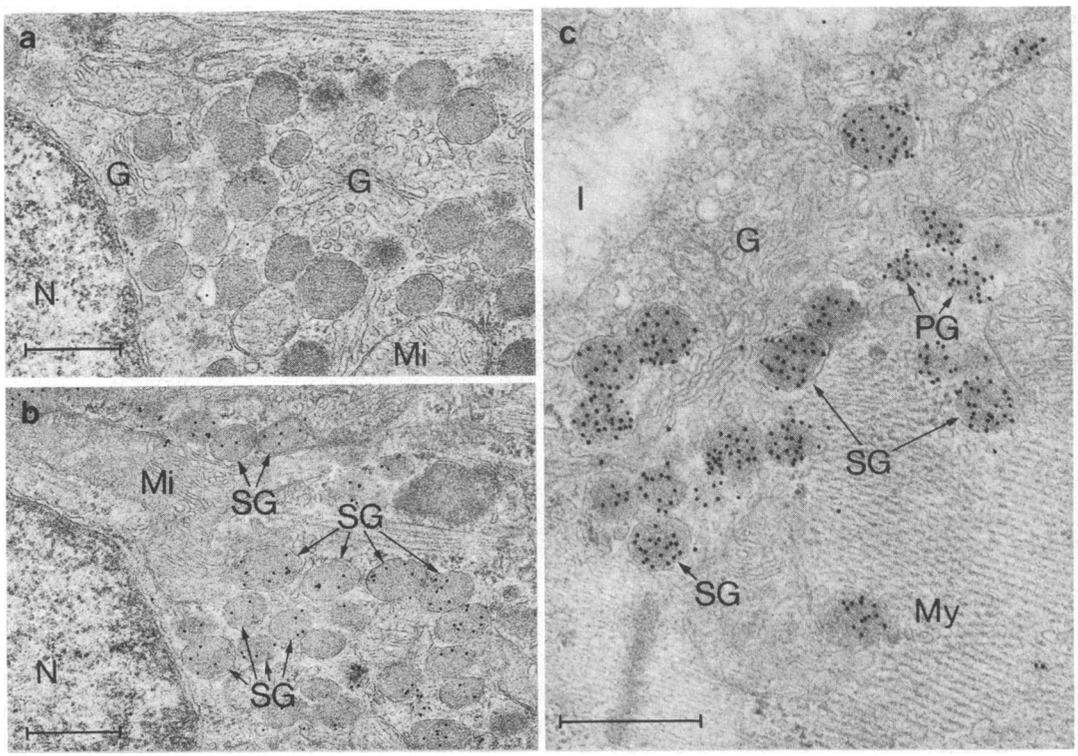

FIG. 6. Immunogold electron microscopy 56 days p.o. of an atrial graft to the muscle bed. (*a*) Perinuclear region of a myocyte in the graft, showing nucleus (N), Golgi apparatus (G) surrounded by numerous secretory granules, and mitochondria (Mi). This negative control was stained with CDD-preadsorbed anti-CDD (1:8000 dilution); some background staining is visible. (*b*) Anti-CDD (1:8000) staining of a similar region as seen in *a*, with exclusive labeling of secretory granules (SG). (*c*) Anti-CDD (1:4000) labeling of SG in the peripheral region of the cell and of granules in formation (PG), with weak labeling of the Golgi apparatus (G). Almost no label is observed in myofibrils (My) and in the interstitial space (I). (Bars = 0.5 μm.)

in the muscle bed (Fig. 1*a*) and at the subcutaneous site (Fig. 2 *a* and *b*). In most grafts to the muscle bed, a sharp borderline was seen between reorganized myocardium and regenerated skeletal muscle fibers (Fig. 1*b*).

There were no significant differences between transplants analyzed between p.o. weeks 3 and 11.

Isomyosin Expression. Frozen sections of transplants were stained with mAbs specific for rat α- and β-type heavy chains of cardiac myosin, respectively. With neonatal rat hearts used as references, anti-α-type myosin mAb stained the atrium with maximal intensity and the ventricle with lesser intensity. In contrast, staining with anti-β-type myosin mAb was confined to the ventricle (Fig. 2 *c* and *d*). A qualitatively similar pattern was obtained with the transplants: atrial grafts stained intensely with anti-α-type myosin mAb but only weakly (though more intensely than authentic atria) with anti-β-type myosin mAb (Figs. 2 and 3). Ventricular tissue, on the other hand, was always stained intensely with the anti-β-type myosin mAb (Fig. 3*d*). Skeletal muscle—i.e., regenerated musculus (m.) tibialis anterior, was negative with anti-α-type myosin mAb, while with anti-β-type myosin mAb some fibers were positive. These were type I slow muscle fibers, as was shown by parallel immunocytochemistry using the m. soleus which contains about 50% type I fibers (Fig. 3*f*). Slow skeletal-muscle myosin and cardiac β-type myosin are immunologically (5) and genetically (15) related or are identical. Controls lacking the first antibody showed some background staining, especially in regions occupied by cardiac muscle (Fig. 3*e*).

Ultrastructure. Ventricular tissue grafted into a skeletal muscle bed displays the ultrastructural features typical for myocardial cells: myofibrils, one nucleus per cell, and intercalated discs connecting neighboring myocytes (18) (Fig. 4*b*). Here we investigated whether atrial grafts would maintain the specific feature of secretory granules (19), the storage organelles for atrial peptide hormones (20–23). While nearly all myocytes in atrial transplants contained granules (Fig. 4*a*, *c*, and *d*), at the most 4% of the ventricular myocytes displayed these structures. The apparent diameters of these granules had a size distribution between those reported for authentic atrial cells of the neonatal (Fig. 4*e*) and the adult rat heart (19).

When atrial and ventricular grafts were examined shortly after transplantation (1 week p.o.) intercalated discs and myofibrils had largely disintegrated and no typical atrial granula were seen.

The data on isomyosin expression and on the distribution of atrial granules are compiled in Table 1.

Presence of Atrial Peptide Hormone. To answer the question whether ectopic atrial grafts contain specific peptide hormones, immunocytochemistry was used at the light (Fig. 5) and electron microscopic levels (Fig. 6). While strong CDD immunoreactivity was found in transplanted atrial tissue (Fig. 5 *b*, *c*, and *e*), ventricular grafts were negative (Fig. 5*f*), as were transplanted atrial controls stained with preimmune serum or anti-CDD antiserum preabsorbed with the immunogen (Fig. 5*a*). The immunoreactive material in most of the transplanted atrial cells was concentrated in the perinuclear region (Fig. 5*c*), where most of the atrial granules (Fig. 4 *a* and *c*) are located. No significant CDD immunoreactivity was found 1 week p.o. in atrial grafts (Fig. 5*d*).

To verify the granular location of the CDD antigen, colloidal gold-conjugated protein A was used as a secondary label for immunoelectron microscopy. Fig. 6 shows the specific (Fig. 6 *a* and *b*) and highly selective (Fig. 6*c*) localization of the gold label in granules of ectopic atrial cells (cf. ref. 26).

We conclude that ectopic atrial myocardium resumes its endocrine function in that atrial peptide hormone is synthesized and stored in secretory granules.

DISCUSSION

We have made use of the surprising longevity of ectopically grafted cardiac tissue (cf. refs. 3 and 18) to study atrial versus ventricular differentiation. To this end, we used isoforms of contractile proteins and cardiac peptide hormone as histochemical markers. In that respect, the "in vivo culture" of grafted atrial and ventricular tissue complements recent investigations on isomyosin expression (27) and atrial hormone production (28) in cell-culture systems. In addition, it allows the study of cell-to-cell and humoral interactions in the environment of a whole organism.

The higher efficiency of minced atrial as compared to ventricular tissue to survive and to reorganize at ectopic sites might be related to the higher capacity of atria to regeneratively respond to injury by cell proliferation (29). Alternatively, atrial tissue might be more potent than ventricular tissue in attracting an efficient vascularization from the host before cells are lost by necrosis.

Regarding the expression of α- and β-type myosin heavy chain genes, it was found that atrial and ventricular myocytes at ectopic sites retain a pattern characteristic of their origin—i.e., near-absence of the β-type myosin heavy chain in atrial grafts and high concentration in ventricular grafts. It appears that the neonatal situation of myosin expression persists, at least in ventricular grafts, in which all cells had remained positive for β-type myosin when stained 5 or 11 weeks after transplantation, whereas ventricular β-type myosin production is reduced postnatally in the rat (14). A low level of β-type myosin heavy chain gene expression in rat atria has been demonstrated by nucleic acid hybridization experiments (15). Thus, a weak β-type myosin heavy chain immunoreactivity (cf. Figs. 2*b* and 3*b*) in ectopic atrial transplants would be within the range of myosin expression of authentic atrial myocardium.

Specific atrial granules are already numerous in the hearts of neonatal rats and remain so throughout life (19). Thus, their presence can be used as a time-independent ultrastructural marker for atrial versus ventricular character of myocytes. Our results show that this regional marker is specifically expressed in the majority of cells in ectopic transplants. In contrast to the situation with ventricular myosin expression, the increase of the granule diameter with time indicates some maturation process of atrial tissue at the ectopic site.

We have demonstrated that the production and storage in specific granules of the atrial peptide hormone cardiodilatin (CDD) persists in long-term grafts and that it is confined to myocytes of atrial origin. Thus, atrial cells are intrinsically programmed to perform their myoendocrine functions irrespective of their anatomical environment.

Since ectopic grafts of cardiac muscle tissue do not impair the viability of the recipient mice, they can be used as models to study aspects of cardiac developmental physiology like vascularization, innervation, plasticity of the differentiated state, and hormonal interactions.

In animal models of heart disease, such as the cardiomyopathic golden hamster (30), environmental influences on degenerative changes and endocrine dysfunction of the atrial myocard might be studied by ectopic transplantation experiments of the kind described in this study. The use of the nude mouse host possibly allows the extension of these studies to human cardiomyopathies.

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