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Chronic Hemodynamic Unloading Regulates the Morphologic Development of Newborn Mouse Hearts Transplanted into the Ear of Isogeneic Adult Mice

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The morphologic development of newborn mouse hearts transplanted into the pinna of the ears of isogeneic adult mice was assessed in comparison to in situ ***ventricular myocardium of recipients. The grafted hearts became vascularized from the auricular artery at the base of the ear, and although these preparations appeared not to be intrinsically innervated, most of them showed grossly visible pulsatile activity. Since they were not subjected to hemodynamic load due to working against a pressure gradient, this technique provided an interesting experimental model for studies on the growth of chronically unloaded tissue. The ultrastructure of the myocardium from neonatal mouse hearts, which were fixed immediately after dissection, revealed no differences in comparison to previously published observations. By 2 months, there was virtually no change in the myocardial cell size as compared with newborn mouse cardiac tissue. The heterotopic hearts showed a mature ultrastructural appearance, with parallel bands of myofibrils alternating with rows of mitochondria and differentiated intercalated discs comparable to*** in situ ***myocardium. The interstitial space was widened due to fibrous tissue, with activated fibroblasts and a few mononuclear cells. In contrast, by 6 months after transplantation, the heterotopic myocardium showed a dispersion of the measured cell diameter of myocytes, with atrophy of a certain population of cells and hypertrophy in others; nevertheless, the mean cell diameter was similar to that observed in 2-month grafts. The myocytes showed significant dissociation from each other in fibrous tissue and a cellular infiltrate composed predominantly of mononuclear cells, and greater variability of the parallel arrangement of cells. They often contained myofibrils coursing in different directions rather than in parallel. Normal-sized or predominantly atrophic degenerated myocytes, characterized by a wide variety of ultrastructural alterations, were present. By 12 months after transplantation, the myocytes of heterotopic hearts were smaller in size in comparison to those after 2 or 6 months. The graft cells were separated from each other by fibrous tissue and mononuclear cells and were not aligned in parallel within the tissue; often, they appeared to have lost their connections with adjacent cells. The myofibrils within cells were strikingly disorganized, coursing in different directions. Severely degenerated myocytes were commonly seen. These results, without precluding the possible role of neural and hormonal stimuli, clearly indicate that hemodynamic work load regulates the developmental growth of newborn mouse heart transplanted into the pinna of the ear of isogeneic adult recipient mice. In other words, the mass of cardiac tissue would be adjusted to meet the prevailing hemodynamic demands. This may have a causal connection to induction and regulation of the cardiac hypertrophic growth in the adult. (Am J Pathol 1992, 141: 183–191)***

Hemodynamic, neural, and hormonal stimuli have been suggested as factors that are involved in the process of cardiac cell growth.[1–5] Studies in surgical models have shown that hemodynamic overloading or underloading results in cardiac hypertrophy or atrophy, respectively.[6,7] These findings have led to the hypothesis that mechanical loading variation functions as an independent regulator of cardiac hypertrophic growth in the adult. To test this hypothesis, cultured adult cardiocytes and isolated

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papillary muscles were submitted to a change in load.[8] Both preparations exhibited increased nuclear and cytoplasmic protein synthesis in response to overload, probably dependent on intracellular sodium influx. Study on neonatal rat cardiocytes in culture could demonstrate that mechanical stimulus induces specific gene expression as well as protein synthesis and cell hypertrophy via protein kinase C activation, without the involvement of humoral factors.[9] Recent investigation on embryonic ventricular implants after 1–14 weeks in the anterior eye chamber of adult host rats showed that myocytes proliferated and differentiated in the absence of hemodynamic load, although cellular hypertrophy growth did not continue after differentiation.[10] The polarized alignment of cells and myofibrils was defective.

The technique of free grafting whole mouse hearts into the pinna of the ear of isogeneic adult recipients provides a simple tool for *in vivo* studies of "isolated" hearts.[11–13] The vascular supply to the graft becomes rapidly established, allowing adequate blood supply and consequent nutritional support of the transplanted organ. Also, the grafts show functional activity, as revealed by grossly visible pulsations or recorded electrical activity for more than 2 years. Although with pulsatile activity, the grafted heart does not perform hemodynamic work. Thus, this study assessed the morphologic development of newborn mouse hearts transplanted into the pinna of the ear of isogeneic adult mice in comparison to *in situ* ventricular myocardium of recipients.

Materials and Methods

One-month-old inbred BALB/c mice of either sex, weighing between 17–23 g at the time of transplantation, served as recipient animals in this study. Newborn mice of the same strain (0–24 hr old) served as cardiac donors. One or both ears of a given recipient were used.

Before cardiac transplantation, recipient mice were anesthetized with ether. After cleansing of the dorsum of the pinna of the mouse ear with 70% alcohol, an incision penetrating only the epidermis, 2–5 mm in length, was made with a scalpel transversal to the longitudinal axis of the ear, 3–4 mm distal to its implantation into the skull. A small pocket between the skin and cartilage was blunt dissected toward the tip of the ear with delicate curved forceps. The total donor heart was excised without pericardial sac and inserted into the ear pocket. Gentle pressure with the tips of the forceps was applied to the ear to express the air from the pocket and facilitate the adherence between donor and recipient tissues. No suture or adhesive was used to close the incision.

The animals were housed in polypropylene cages, maintained under controlled conditions, and given free access to laboratory chow and tap water; 2, 6, and 12 months after transplantation, the mice were sacrificed in light ether anesthesia by exsanguination from the abdominal aorta.

At the end of each experimental period, six ears containing the grafts that developed grossly visible pulsations were fixed by immersion for 24 hours at room temperature in a solution containing 2% paraformaldehyde plus 2.5% glutaraldehyde. Approximately 70% of heart grafts showed grossly visible pulsatile activity when examined with a stereomicroscope at fourfold magnification. After fixation, all ears were sectioned transversally across the graft at 2-mm intervals. The tissues were then dehydrated in ascending concentrations of ethanol, embedded in glycol methacrylate, sectioned at 2 μm, stained with toluidine blue and basic fuchsin,[14] and examined under the light microscope. Six hearts from newborn (0–24 hr) mice were also processed. The weights of the heterotopically transplanted hearts were not obtained since the "fused" borders of the grafts were continuous with the surrounding subcutaneous tissue. The atrial and ventricular chambers could not be separated after long-term transplant.

At the end of each experimental period, the respective *in situ* hearts were rapidly removed, rinsed in ice-cold 0.9% NaCl, and sectioned across the ventricles. Midventricular sections, 2 mm thick, were processed as mentioned.

The representative diameter of muscle fibers was measured with the help of camera-lucida images projected from preparations on a digitizer tablet interfaced to a central processor (Morphomat 10, Carl Zeiss, Germany). Areas containing a properly oriented cross-section of myocytes were selected from each section (excluding papillary muscles and subendocardium), and the minor diameter was measured in each 200–250 fibers per heart. The number of measurements was chosen arbitrarily. Previous studies offered guidance on this point, although they have varied widely. For example, 20 determinations per case,[15] 100–150,[16,17] and 200 or more[18–20] per heart have been accepted. A 25-point ocular (Integrating eyepiece II, Carl Zeiss, Germany) was used to determine the volume fraction of cellular and extracellular components by counting all points lying over myocytes and interstitium, respectively. At least 18 fields from each rat were examined at ×200 magnification in randomly selected sections, without respect for the direction of sectioning. The cellular-to-extracellular components ratio was calculated as: volume fraction of myocytes divided by volume fraction of interstitium. All morphometric data were collected blindly, and the code was broken at the end of the experiment.

Samples of pulsatile heterotopic and *in situ* hearts (from the left ventricular-free walls, halfway between the base and apex) were rapidly excised for electron micro-

scopic study from three animals at the end of each experimental period. Three hearts from newborn (0–24 hr) mice were also processed. Small blocks of tissue less than 1 mm in diameter were fixed by immersion in 2.5% glutaraldehyde buffered in phosphate (pH 7.3) for 3 hours at 4°C. After rinsing and washing in buffer, tissues were postfixed for 2 hours in ice-cold 1% osmium tetroxide buffered in phosphate, dehydrated in ascending concentrations of acetone, and embedded in araldite. Semithin sections (0.5 μm) stained with toluidine blue were examined under the light microscope, and a suitable area was selected for preparations of ultrathin sections. These were obtained on a Sorvall MT-5000 ultramicrotome (Du Pont Company, Newtown, Conn, USA), doublestained with uranyl acetate and lead citrate, and examined with a Zeiss EM 109 electron microscope at 80 kv.

Data were analyzed using a Statgraphics statistics program (Graphic Software Systems, Inc., USA) for the IBM PC computer (Monydata Teleinformática Ltd., S.P., Brazil). Comparisons of the means of the measured cell diameter of myocytes from heterotopic and *in situ* hearts at each experimental period were made by one-way analysis of variance. One value for each case entered into the analysis (the number of hearts in each group at each experimental period was 6). A level of significance of 5% was chosen to denote difference between group means. These data were presented as box and wisker plot graphs. The box and wisker graph shows the body of data, being especially useful for comparing several batches of data.[20] The box ends at the lower and upper fourth (closely related to the interquartile range) and contains a crossbar at the median. The line from each end of the box represents the most remote point. Regression analysis was used to evaluate the cellular-to-extracellular components ratios from both groups for each experimental period. Significance was accepted at the 5% probability level. These data are reported as mean ± SEM.

Results

Although the measured diameter of myocytes from heterotopic hearts did not change statistically from 0 to 6 months after transplantation, there was a distinct broader distribution of cell size at 6 months, with an augmented number of small myocytes. Significantly, cell diameter markedly decreased by 12 months (Figure 1). This may be due, at least in part, to a difference between atrial and ventricular cells. Contrarily, myocardial cell diameter of *in situ* hearts significantly increased from 2 to 12 months (Figure 2).

The cellular-to-extracellular components ratio in heterotopically transplanted hearts declined significantly with time. In contrast, in the *in situ* hearts, there was virtually no change in the cellular-to-extracellular ratio (Figure 3).

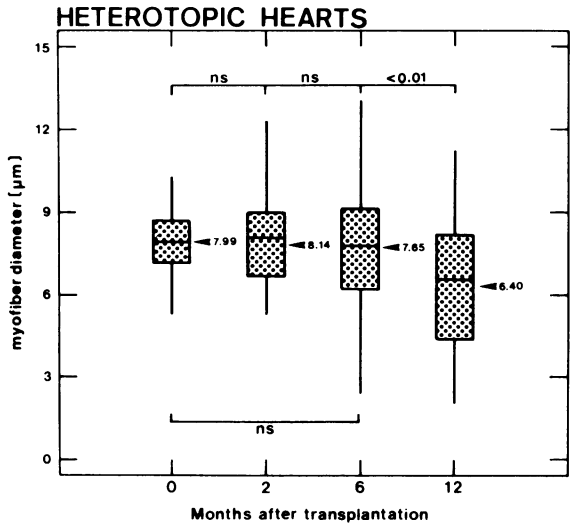

Figure 1. *Boxplots for the minor diameter of myocytes of heterotopic hearts. Cell diameter decreased markedly by 12 months. Although the measured diameter of myocytes did not change statistically from 0 to 6 months, there was a distinct broader distribution of cell size at 6 months, with augmented number of small myocytes. Arrowheads indicate the means (number of hearts at each experimental period = 6; F = 13.45; d.f. = 23).*

The size of myocardial fibers and the volume fraction of cellular and extracellular components were assessed with light microscopy morphometry. Light microscopy sampling in morphometry was preferred because large test areas could be analyzed. The use of thin plastic sections and combined staining with toluidine blue and basic fuchsin allowed adequate resolution of structural details.

The electron microscopic appearance of *in situ* hearts 2, 6, and 12 months after transplantation did not differ

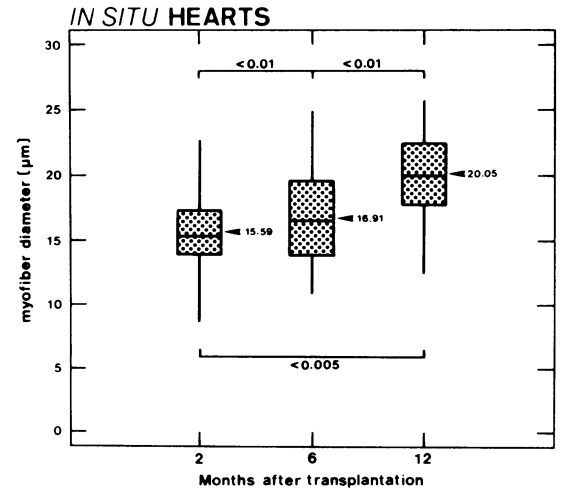

Figure 2. *Boxplots for the minor diameter of myocytes of* in situ *hearts. Myocardial cell diameter increased significantly from 2 to 12 months. Arrowheads indicate the means (number of hearts at each experimental period = 6; F = 16.39; d.f. = 17).*

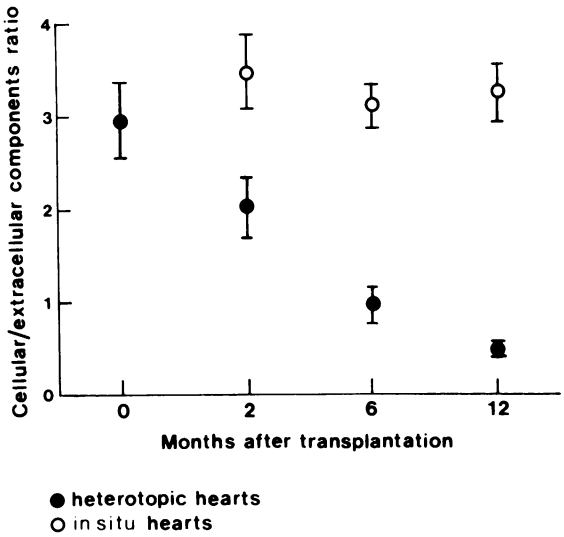

Figure 3. *Cellular-to-extracellular components ratio (volume fraction of myocytes divided by volume fraction of heterotopic and* in situ *hearts) determined from the same material used in Figures 1 and 2. Data are mean ± sem (heterotopic hearts: number of hearts at each experimental period = 6; F = 16.36; d.f. = 23; and* in situ *hearts: number of hearts at each experimental period = 6; F = 0.32; d.f. = 17).*

from that reported in the literature.[21] A uniform arrangement of myofibrils parallel to each other could be seen. Z bands dividing the sarcomeres were linear and perpendicular to the myofilaments. Myofibril bands alternated with rows of mitochondria. Sarcoplasmic vesicles and glycogen were present in the intermyofibrillar space. The intercalated discs of the myocardium appeared normal, moderate, or markedly folded. Vascular and interstitial connective tissue elements were present.

The myocardium of newborn mice showed myocytes as oval or slightly elongated, containing sparse definite striated myofibrils with clearly identifiable Z bands and actin and myosin myofilaments forming clear A, I, and H bands; however, no M bands were noted. The nuclei of these cells were oval and large in comparison to the cells as a whole and contained one or more prominent nucleoli. Mitotic figures were seen in myocytes of neonatal mouse heart. Occasional small groups of myofilaments in disarray were scattered randomly in the cytoplasm. Mitochondria were rather sparse and small as compared to the adult ventricular myocardial cell. Sarcoplasmic vesicles and glycogen were present in the cytoplasm. A great number of lipid droplets could be detected. The intercalated discs were immature. Adherens junctions (desmosomes and fascia adherens) were clearly seen, whereas gap junctions were not identified (Figures 4, 5).

By 2 months after transplantation, elongated myocardial cells with abundant myofibrils were seen. In most of the areas, myocytes were arranged in parallel bundles. The sarcomeres were well developed, with aligned myofilaments, similar to *in situ* myocardium. The nuclei were

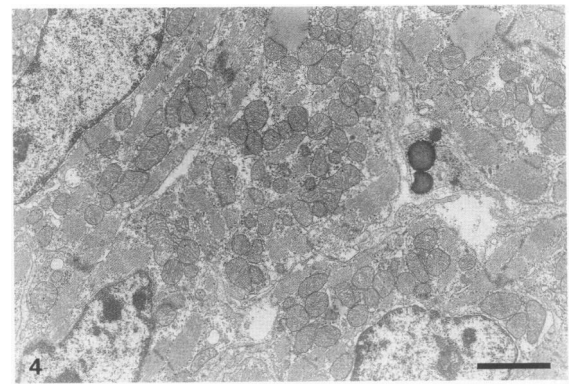

Figure 4. *Myocardium of newborn mice. Myocytes contain sparse striated myofibrils. Mitochondria are sparse and small. Sarcoplasmic vesicles and glycogen are present in the cytoplasm. A great number of intracellular lipid droplets can be seen. Bar = 2 μm.*

elongated and smaller than those of newborn myocytes in comparison to the cell as a whole. Two-month transplanted myocardium had no mitotic figures. The mitochondria had the adult pattern of cristae and were distributed in rows among the myofibrils, similar to the arrangement observed in *in situ* hearts. However, an increased number of mitochondrial amorphous dense granules occurred. A few lipid droplets were present in the cytoplasm of each cell. The intercalated discs, composed of desmosomes, fascia adherens, and small gap junctions, appeared as mature structures. The interstitial space was diffusely widened, particularly around vessels, due to collagen fibrils and activated fibroblasts and areas of mononuclear cells (Figures 6, 7).

By 6 months after transplantation, the arrangement of myocardial cells in parallel bundles was lost. Small cardiac cells, containing few myofibrils, were occasionally associated with larger myocytes. In contrast to the myocardium 2 months after transplantation, there was a prominent disorganization of the orderly array of the con-

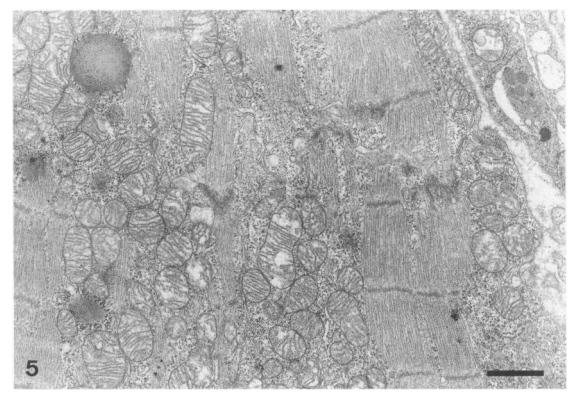

Figure 5. *Myocardium of newborn mice. The intercalated disc is immature. Adherens junctions (desmosomes and fascia adherens) are seen clearly whereas gap junctions are not identified. Mitochondria are swollen. Note clearly identifiable Z, A, and I bands, but absence of M bands. Bar = 1 μm.*

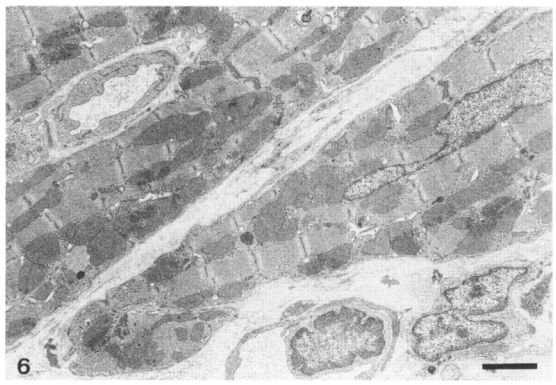

Figure 6. *Myocardium 2 months after transplantation. Elongated myocardial cells, with abundant myofibrils, arranged in parallel bundles are seen. Rows of mitochondria alternate with myofibril bands. The nuclei are elongated. The interstitial space is widened due to collagen fibrils, and contains activated fibroblasts and a great number of vessels. Bar = 2 μm.*

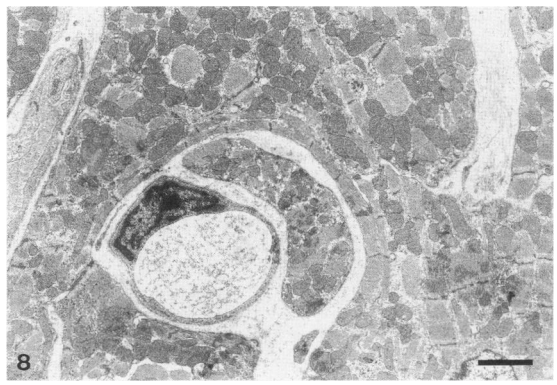

Figure 8. *Myocardium 6 months after transplantation. The arrangement of myocardial cells in parallel is lost. Small myofibrils, containing few myofilaments, are associated with larger myocytes. The orderly array of the contractile elements is disturbed. Mitochondria are scattered in the cytoplasm of the myocytes and show an increased number of amorphous dense granules. Bar = 2 μm.*

tractile elements, with myofibrils coursing in different directions within a given cell. The nuclei were elongated and proportional to the cell size as a whole. Although the mitochondria were morphologically similar to those observed 2 months after transplantation, they were scattered in the cytoplasm of myocytes with an increased number of intramitochondrial granules. Widened intercalated discs with desmosomes and occasional fascia adherens junctions were observed. Gap junctions, however, were not identified. The myocardial cells were markedly separated from adjacent cells by fibrous tissue and an infiltrate of mononuclear cells, predominantly macrophages. A certain population of normal-sized or atrophic cells showed severe degeneration characterized by marked loss of contractile elements, cytoplasmic vacuolization, mitochondria of various sizes, and prominent accumulation of myelin figures, lisosomes, and fragmented membranes. Fatty infiltration was also noted at the central cavity and on the periphery of the grafts (Figures 8, 9).

By 12 months after transplantation, the electron microscopic appearance was qualitatively similar to that observed after 6 months. However, some changes were more pronounced, particularly: (a) small cardiac cells were markedly dissociated from adjacent myocytes by large amounts of fibrous connective tissue; (b) most of the cells joined together by small junctional structures that were formed by the apposition of two areas of the plasma membrane of distinct myocytes; (c) increased number of myocardial cells showed severe degenerative changes; (d) pronounced presence of perivascular focal areas of mononuclear cells, predominantly macrophages; and (e) marked diffuse fatty infiltration (Figures 10–14).

Abundant vascular elements were identified in the myocardium 2, 6, and 12 months after transplantation. Although nonmyelinated nerves were found on the graft surface, nerve tissue was not observed inside the myocardial tissue, i.e., the transplanted hearts appeared not to be intrinsically innervated. Atrial cells growing in the ear, identified by secretory granules, were not analyzed.

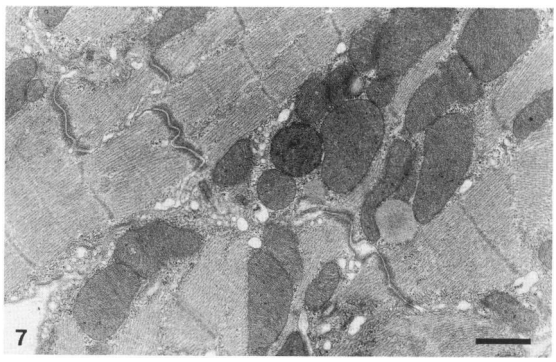

Figure 7. *Myocardium 2 months after transplantation. Mature intercalated disc composed of desmosomes, fascia adherens, and small gap junctions. The sarcomeres are well developed. An increased number of small amorphous dense granules can be observed in the mitochondrial matrix. Bar = 1 μm.*

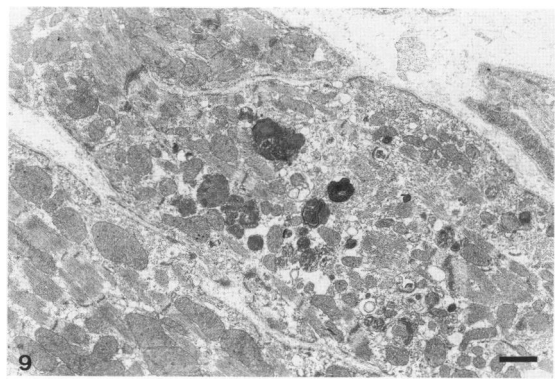

Figure 9. *Myocardium 6 months after transplantation. A widened intercalated disc is seen. Desmosomes and fascia adherens are seen clearly although gap junctions are not identifiable. A markedly degenerated myocyte is seen. Bar = 1 μm.*

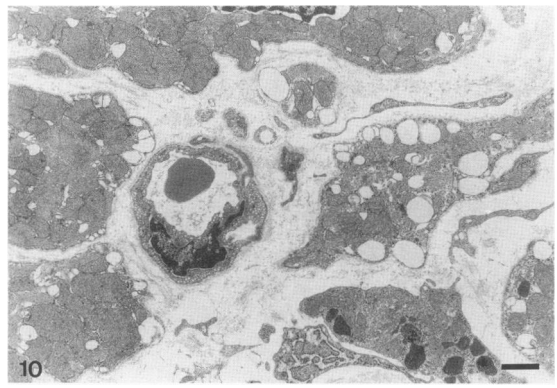

Figure 10. *Myocardium 12 months after transplantation. Small cardiac cells are markedly dissociated from adjacent myocytes by large amounts of fibrous connective tissue. Bar = 1 μm.*

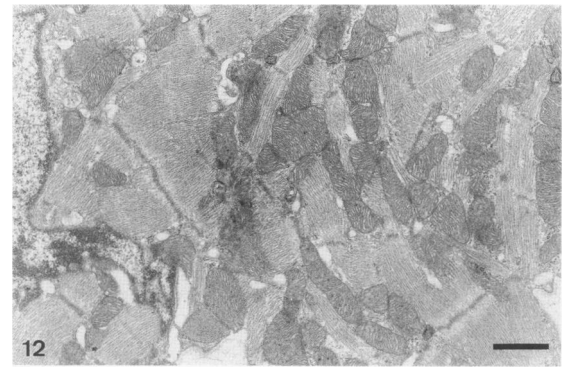

Figure 12. *Myocardium 12 months after transplantation. Prominent disorganization of the orderly array of the contractile elements, with myofibrils coursing in different directions within a given cell. Mitochondria show an increased number of small amorphous dense granules. Bar = 1 μm.*

Discussion

Newborn mouse hearts transplanted into the pinna of the ear of the isogeneic adult recipient mouse became vascularized from the auricular artery at the base of the ear, and although these preparations appeared not to be intrinsically innervated, most of them showed grossly visible pulsations. Since the transplanted hearts were not subjected to hemodynamic load due to working against a pressure gradient, this technique provided an interesting experimental model for studies on the growth of chronically unloaded heart tissue. The central cavities of the grafts were filled with adipose tissue (fatty infiltration).

The ultrastructure of the myocardium from neonatal mouse hearts that were fixed immediately after dissection revealed no differences in comparison to previously published observations.[22] By 2 months, heterotopic hearts showed a mature ultrastructural appearance, with parallel bands of myofibrils alternating with rows of mitochondria and differentiated intercalated discs comparable to *in situ* myocardium. However, the myocardial cell size did not change as compared to newborn mouse cardiac tissue. Besides, the interstitial space was widened due to fibrous tissue, with activated fibroblasts and a few mononuclear cells. In contrast, by 6 months after transplantation, the heterotopic myocardium showed a dispersion of the measured cell diameter of myocytes, with atrophy of a certain population of cells and hypertrophy in other; nevertheless, the mean cell diameter was similar to that observed in 2 month grafts. The myocytes showed significant dissociation from each other by fibrous tissue and a cellular infiltrate composed predominantly of mononuclear cells, and generalized variability of the parallel arrangement of cells. They often contained myofibrils coursing in different directions rather than in parallel. Normal-sized or, predominantly, atrophic degenerated myocytes, characterized by a wide variety of ultrastructural alterations, were present. By 12 months after transplantation, the myocytes of heterotopic hearts were smaller in size in comparison to those after 2 or 6 months. The graft cells were separated from each other by fibrous tissue and mononuclear cells, and not aligned in parallel within the tissue; often, they appeared to have lost their connections with adjacent cells. The myofibrils within cells were

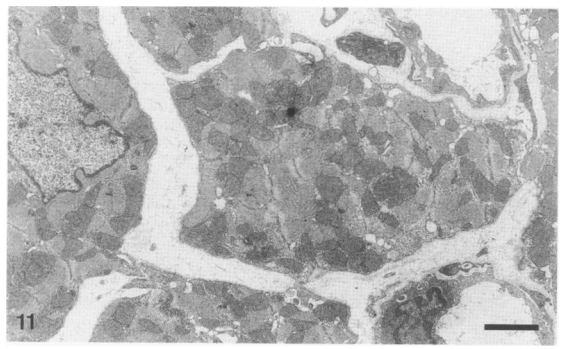

Figure 11. *Myocardium 12 months after transplantation. Most of the cells join together by small junctional structures formed by the apposition of the plasma membrane of distinct myocytes. Bar = 2 μm.*

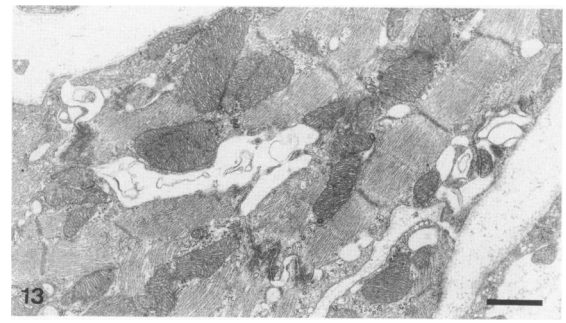

Figure 13. *Myocardium 12 months after transplantation. Widened intercalated disc with desmosomes and fascia adherens junctions are seen. Gap junctions are not seen. Bar = 1 μm.*

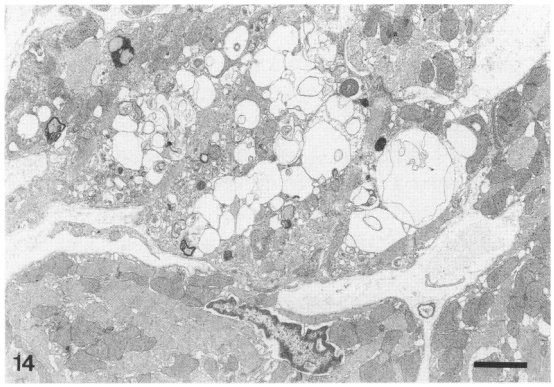

Figure 14. *Myocardium 12 months after transplantation. Myocardial cells show severe degenerative changes. Bar = 2 μm.*

strikingly disorganized, coursing in different directions. Severely degenerated myocytes were commonly seen.

Three possibilities can be considered to explain the changes observed in the heterotopically transplanted newborn mouse hearts. These changes could be due to 1) humoral influences directly affecting the rate of myocardial protein synthesis, 2) changes in the cardiac work load, and 3) influences in the environment of the ear.

Among the various suggested circulating growth stimuli, catecholamines and angiotensin peptides may play a central role.[1,4,5,23–37] Besides, a number of circulating substances such as thyroid hormone, adrenocorticoids, insulin, or growth hormone may have a direct or permissive trophic effect on the myocardium.[25,38–41] The model studies in the present investigation, as previously commented, consider a vascularly perfused, spontaneously beating, essentially unloaded heart exposed to the same circulating substances as the *in situ* heart.

Another possible influence on myocardial cell size and ultrastructure, relevant to the present study, is decreased hemodynamic load. Studies on adult mammalian myocardial cells cultured on a laminin-coated silicone membrane and isolated quiescent and contracting papillary muscle preparations have recently suggested that cell deformation appears to be a sufficient stimulus for the induction of cardiac hypertrophy growth, as revealed by an increase in [^{3}H]uridine incorporation into nuclear ribonucleic acid, a significant increase in total nuclear ribonucleic acid content, and a significant increase in [^{3}H]phenylalanine incorporation into the cytoplasmic protein.[8] The stimulatory effect of load on protein synthesis would be due to sarcolemmal deformation with consequent entry of sodium and cell growth stimulation. More recently, it could be shown that mechanical stimuli by stretching cultured rat neonatal adherent myocytes induce specific protooncogene stimulation and synthesis of proteins via activation of protein kinase C.[9] Another point to be considered is experimental observations showing that nonadherent unloaded adult cat ventricular myocytes maintained in serum-free culture medium compared with substrate-adherent loaded cells rapidly lost their differentiation of form and function.[42] The newborn hearts transplanted into the pinna of the ears of adult mice maintain an active tension during contraction. Even without hemodynamic load involving shortening or isotonic work, i.e., "external work," the myocytes of the grafts advanced to a mature contractile phenotype, whereas prolonged culture of myocytes resulted in loss of structure. Although it could be suggested that this cell differentiation is a humoral effect since the heterotopic hearts were beating, the heart may undergo "internal work" through isometric work load.

The environment of the ear raises two questions. The first is the failure of perfusion through the coronary circulation. In-growth of blood vessels may not perfuse the myocardium in the normal manner. The second is the influence of the local milieu. The fibroblasts and their elaboration of cytokines and growth factors could have substantial influence on the heart. In this context, the interface between the ear and the graft could not be distinguished, i.e., the "fused" borders of the grafts were continuous with the surrounding subcutaneous tissue.

The intercalated discs observed in the myocardium 6–12 months after transplantation into the ear showed various degrees of dissociation. Although the adherens junctions (fascia adherens and desmosomes) were present and preserved, the gap junctions or nexuses shared between adjoining cardiac cells were markedly separated, disrupted, or even absent, particularly after 12 months. In this later condition, most of the cells contained structures formed by the apposition of two areas of the sarcolemma of two adjacent cells. The intercalated discs 2 months after transplantation had a mature structure, but with gap junctions small in size and with low complexity. Since the gap junctions are the membrane specializations that permit the exchange of small metabolites and ions and the spread of electrical excitation between neighboring cells,[43] these junctions would be impaired step-by-step in the transplanted hearts, thus contributing to the gradual atrophy and degeneration of myocytes.

In summary, the results of this study, without precluding the possible role of neural and hormonal stimuli, clearly indicate that hemodynamic work load regulates the developmental growth of newborn mouse heart transplanted into the pinna of the ear of isogeneic adult recipient mice. In other words, the mass of cardiac tissue would be adjusted to meet the prevailing hemodynamic demands. This may have a causal connection to induction and regulation of cardiac hypertrophic growth in the adult.

Acknowledgments

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