Microtubules and Desmin Filaments During Onset of Heart Hypertrophy in Rat: A Double Immunoelectron Microscope Study


The distribution of tubulin and desmin, the constituent proteins of microtubules and intermediate filaments, respectively, were studied in normal and hypertrophied rat myocardium by high-resolution immunofluorescence and immunoelectron microscopy. Cardiac hypertrophy was induced in 25-day-old rats by aortic stenosis. In the normal heart, double immunolabelling of ultrathin frozen sections of papillary muscle using gold-labelled probes for tubulin and desmin showed that microtubules ran primarily in a longitudinal direction through the intermyofibrillar spaces, perpendicularly to the desmin filaments. Microtubules were present near nuclei, mitochondria, and plasma membranes, while desmin filaments formed transverse connections between adjacent Z disks. No tubulin was observed near the intercalated disks, which were rich in desmin filaments. In hypertrophied hearts, myocytes exhibited the typical morphological features of developing hypertrophy. While there was little difference in the distribution of the microtubules around mitochondria and at the plasma membrane, considerable increases were seen near the nuclei and along the myofibrils. Desmin labelling was distributed transversely as in the controls; however, sometimes it was longitudinally oriented either in the intermyofibrillar space linking 2 Z disks out of register or along digitations of the intercalated disks connecting neighboring desmosomes. The unique rearrangement of desmin and tubulin filaments in hypertrophied cardiac myocytes emphasizes their distinct role in myocyte organization. We suggest that, during the development of cardiac hypertrophy, desmin filaments are mainly involved in maintaining the myofibrils in register, whereas the degree of assembly of microtubules is correlated with the rate of protein synthesis and with myofibrillogenesis. (Circulation Research 1987;60:327-336)

Myocyte hypertrophy, an adaptive response of the heart to a pressure overload, is accompanied by a coordinated accumulation and/or alteration of cellular components such as myofibrils, mitochondria, and sarcoplasmic reticulum. Little information is available on the mammalian heart with regard to the structures involved in cellular organization and the process of myocyte enlargement. Two cytoskeletal elements, microtubules and intermediate filaments (for review see Weber and Osborn) were reported to be involved in the assembly of sarcomeres during myogenesis in cultured myoblasts and skeletal muscle and during the postnatal development of cardiac muscle. Thus far, studies of the cytoskeleton elements in hypertrophied myocardium have been restricted to descriptions of increases in the amount of microtubules or intermediate filaments in various types of hypertrophy. In our previous investigation, carried out on the distribution of tubulin and desmin in isolated myocytes during cardiac hypertrophy, we found a transient reorganization of the microtubule pattern in a subpopulation of rat myocytes during the onset of cardiac hypertrophy induced by pressure overload. No significant alteration was concomitantly observed in the pattern of desmin filaments. However, the poor resolution obtained with isolated cells about 30 μm thick precluded the visualization of microtubule and/or intermediate filament interactions with the membranes or the organelles, the latter being known to vary significantly during cardiac hypertrophy in the adult rat.

In the present study, we investigated in more detail the changes in distribution of tubulin and desmin during cardiac hypertrophy induced by pressure overload. The distribution of tubulin and desmin was examined 7 days after aortic stenosis. At this time, the ultrastructural changes that accompany the development of myocyte hypertrophy are evident and a maximum number of myocytes exhibit alterations in their pattern of microtubule organization. In this study, we have improved the visualization of cytoskeletal structures in striated muscle by using a technique for immunocytochemical labelling of ultrathin frozen sections (80 nm) that allows precise localization of specific intracellular proteins while preserving the integrity of the tissue. The distribution of tubulin and desmin were examined in ultrathin sections of papillary muscle by double indirect immunolabelling either by light microscopy, using two different fluorescent antibodies, or by electron microscopy, using two sizes of gold particles (5 nm and 15 nm) as electron-dense markers coupled to the secondary antibodies. The high specificity of the...
labelling combined with the high resolution of electron microscopy (0.4 nm) allowed us to accurately identify the distribution in cardiac myocytes of components that are either present at very low concentration (e.g., microtubules) or difficult to identify by size only (e.g., 10 nm desmin filaments).

The results obtained revealed that the respective changes in distribution of desmin filaments and microtubules were related to different ultrastructural changes that occur during the development of cardiac hypertrophy.

Preliminary results have been presented in the form of an abstract.22

Materials and Methods

Cardiac Hypertrophy

Thoracic aortic stenosis was induced in 25-day-old female Wistar rats (Iffacredo, Lyon, France) by placing Weck hemoclips around the ascending aorta, according to Bugaisky et al.23 These rats were then paired with sham-operated rats of the same litter. Animals were fed ad libitum on M-biscuits.

The sham-operation procedure consisted of ascending aorta dissection without banding.

Tissue Fixation and Section Procedures

Seven days after surgery, sham and operated rats with the same body weight (90 g ± 5) were anaesthetized with 0.15 ml (6%) sodium pentobarbital (Cln Midy). Hearts were arrested by intravenous injection of 0.50 /u,g procaine and immediately fixed in situ at room temperature by aortic perfusion with 2% paraformaldehyde and 0.2% glutaraldehyde in 0.1 M phosphate-buffered saline (PBS), pH 7.2. Hearts were then stabilized with 0.15 ml (6%) sodium pentobarbital (Clin Midy). These rats were then paired with sham-operated rats of the same litter. Animals were fed ad libitum on M-biscuits.

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Immunocytochemical Procedures

Up to the second antibody step, the same immunocytochemical procedure was applied to all sections examined by light or electron microscopy.

Once the sucrose had been removed, sections were washed for 10 minutes with 80 mM ammonium chlo-

ride and then twice in 0.1 M PBS containing 0.5% bovine serum albumin (BSA) and 0.15% glycine. This buffer was used for all washes and to dilute all antibodies. Sections were then incubated for 30 minutes at room temperature with a mixture of anti-α-tubulin and anti-β-tubulin monoclonal antibodies (1/1) (Amer-Sham, Les Ulis, France), washed 6 times for 5 minutes each and then incubated in the second antibody. For electron microscopy, a goat antimouse antibody was used, coupled with small (5 nm) gold particles (Janssen Pharmaceuticals, Beerse, Belgium), whereas a Texas red-coupled antibody of the same type (Janssen) was used for immunofluorescence. After a further wash, sections were incubated with a polyclonal antibody against desmin (kindly provided by V. Small and J. de Meys), washed again, and labelled with either a goat antirabbit antibody coupled to large (15 nm) gold particles (Janssen) or fluorescein-coupled goat antirabbit antibodies (Miles Laboratories, Paris, France). The sections processed for fluorescent microscopy were washed once more in PBS, BSA/glycine, and mounted with coverslips in Gelvatol (Monsanto). Those processed for electron microscopy were washed another 6 times for 5 minutes each, this time in distilled water (pH adjusted to 7.5), counterstained with uranyl oxalate for 8 minutes at pH 7.5 and then with uranyl acetate for 2 minutes and mounted in a thin film of 1.25% methylocellulose (Fluka, Buchs, Switzerland).

Light microscopy was conducted with a Leitz Dialux microscope (Rueil-Nalmaison, France) equipped with epifluorescence optics and Ploemopak filter sets (N0 for Texas red fluorescence, I0 for fluoresceine fluorescence), and electron microscopy with either a Siemens Elmiskop 101 or a Zeiss EM 109 microscope.

Better immunolabelling was obtained if the antibody to be labelled with the smaller gold particles was applied first, in this case the antitubulin antibody. It was verified that there were no differences between the results for sham-operated rats and nonoperated control animals.

Results

Double Immunofluorescent Labelling of Desmin and Tubulin Visualized by Light Microscopy

In sham-operated animals, desmin immunolabelling, when visualized by light microscopy, revealed transverse striations extending across the myocyte (Figure 1a), these striations corresponded to the Z disks in phase microscopy (not shown). The labelling was also abundant close to the intercalated disks extending away in a perpendicular fashion (Figure 1a). In the same section, tubulin labelling (Figure 1b) was concentrated in a ring around the nuclei and was evident as punctate elements or short strands of no particular orientation in the cytoplasm.

In the hypertrophied hearts, the distribution of desmin and tubulin in the myocytes was strikingly altered when compared to that seen in the sham-operated animals (Figure 1c and 1d). As in controls, the desmin labelling generally showed a transverse striated pattern in most myocytes. However, in some myocytes, inter-
medial filament bundles were seen to stretch longitudinally within the myocyte. In addition, more extensive desmin staining developed irregularly at the level of the intercalated disks (Figure 1c). The pattern of tubulin staining (Figure 1d) in some myocytes closely resembled that found in control animals, as shown in Figure 1a. However, in adjacent cells tubulin staining was more intense and seen as thick longitudinal cables (Figure 1d).

In conclusion, distinct differences were seen in the distribution of desmin and tubulin, as visualized by light microscopy, in the hypertrophied rat heart compared to the normal heart. During hypertrophy both networks underwent unique alterations, which occurred in a random manner, resulting in a heterogeneous pattern of desmin and tubulin distribution among the myocytes.

Desmin and Tubulin Immunolabelling Visualized by Electron Microscopy

In sham-operated hearts, desmin labelling (15 nm, large gold particles) was transversely distributed across the intermyofibrillar spaces and was evident at the periphery of Z disks but never penetrated the disks themselves (Figure 2a). However, in sections that grazed the surface of myofibrils, desmin labelling appeared to traverse the outer portion of the disk (Figures 2a–2c). Except for occasional lateral extensions from the Z-disk region to adjacent mitochondria, very little desmin labelling was seen along the myofibrils or close to mitochondria (Figures 2a and 2d). Most of the tubulin labelling (5 nm, small gold particles) was longitudinally oriented in the intermyofibrillar spaces, often close to mitochondria (Figures 2a and 2d). This staining sometimes extended longitudinally over several sarcomeres, passing through the clusters of desmin filaments located at the Z disk (figure 2d). Occasionally, on sections that grazed the periphery of the myofibril and on which desmin labelling circumscribed the Z disk, tubulin staining was either closely parallel to that of desmin (Figure 2c) or was located near a part of the sarcoplasmic reticulum (Figure 2b). Consequently, microtubules sometimes were seen surrounding a Z disk and a sarcomere, though as a rule only a few
microtubules were observed in the neighborhood of myofibrils (Figure 2).

In hypertrophied hearts, the changes in desmin distribution observed by light microscopy were often found to occur in cells displaying a loss of myofibrillar register. In these regions, desmin labelling (large gold particles) was distributed longitudinally between two laterally displaced Z disks (Figure 3a). Alterations in desmin distribution were also associated with disorganization of the sarcomeric architecture where desmin filaments formed arrays connecting the Z disk, mitochondria, and I-band (Figure 3b). The more intense tubulin staining observed by light microscopy throughout the sarcoplasm (Figure 1d) was correlated in electron microscopy with an increased number of microtubules in some myofibrillar areas. These alterations were observed in numerous and random sections (Figure 3). Microtubules were seen either longitudinally apposed to sarcomeres (Figure 3b) or in areas showing disorganized sarcomeres (Figure 3c). In these sections, the microtubules were distributed around the mitochondria and parts of the sarcoplasmic reticulum. However, when studied in a large number of random sections the relative number of microtubules along the mitochondria was found to be the same in both the hypertrophied and the control hearts. No microtubules were observed in the areas where the myofibrils were out of register (Figure 3a).

In Figure 4a (sham-operated) and 4b (hypertrophied animals) the desmin labelling, discretely contained in the Z-disk area, showed no particular relation to the nucleus, even when myofibrils abutted the nuclear profile. In sham-operated rat myocytes, a linear array of microtubules (small gold particles) ran parallel to the

FIGURE 2. This and following figures are electron micrographs of longitudinal ultrathin frozen sections of rat papillary muscle, double labelled with tubulin antibodies (small gold particles) and desmin antibodies (large gold particles). In sham-operated rats, desmin labelling is located between the Z disks. Longitudinal microtubules decorated by the small gold particles (arrows) are located close to the mitochondria (Panels a and d). In sections that graze the myofibril, they may be parallel to desmin staining (Panel c) or run sinusoidally across the fibers (Panel b) near the sarcoplasmic reticulum (arrowhead). (Panel a: ×20,000, Panel b-d: ×43,500.)
nuclear envelope and sometimes extended along many sarcomeres but displayed no apparent connection with the myofibrils (Figure 4a). In hypertrophied myocytes, a large number of microtubules were seen streaming away from the nuclei; some microtubules with curved profiles ran in various directions towards the myofibrils (Figure 4b).

Figure 5 shows that desmin labelling at the plasma membrane was restricted to the periphery of the Z disks that seemed to be anchored to the membrane with unlabelled filaments. On the other hand, tubulin staining parallel to the plasma membrane was sometimes tightly apposed to a mitochondria.

In the intercalated disks of sham-operated rat myocytes, the desmin labelling was concentrated in the region of the desmosomes (Figure 4a). Microtubules were absent from this area and very little tubulin staining was seen in the intermyofibrillar spaces. In hyper-
trophied myocytes, the intercalated disks were found to be altered to various degrees, sometimes displaying deep convolutions (Figure 6b and 6c). As in normal myocytes, the desmin labelling was concentrated in the desmosome region but was more pronounced at the folds of the plasma membrane, sometimes linking two desmosomes (Figure 6b) or joining the desmoplaque to the adjacent Z disk, extending along the entire length of a sarcomere (Figure 6c). Microtubules were still absent from this area.
It is worth noting that, except in a few cases (Figures 2d and 4a), tubulin and desmin were rarely codistributed, suggesting that there were few connections between microtubules and desmin filaments in sham-operated as well as hypertrophied myocytes, even though significant changes occurred in the distribution of these 2 cytoskeletal elements.

Discussion

The purpose of this work was to study, in normal and hypertrophied cardiac muscle, the distribution of microtubules and intermediate desmin filaments believed to contribute to the structural organization and function of striated muscle. The experimental model of myocyte hypertrophy following aortic stenosis in the 25-day-old rat has already been described and discussed. Papillary muscle was chosen for investigation since it constitutes the least heterogeneous area of the heart and adapts rapidly to pressure overload. Thus, it may be hypothesized that the changes observed must be indicative of even more pronounced changes in the rest of the ventricle. The distribution of microtubules and intermediate filaments in heart have each been well documented by electron micro-
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Figure 5. A microtubule, visible near the plasma membrane, seems to connect mitochondria and sarcomeres to plasma membrane (thin arrows). Note, however, presence of unlabelled and unidentified filaments between the Z disk and the plasma membrane (arrow) (×43,500).

We chose a double immunolabelling approach using both light and electron microscopy to gain more insight into the distribution of these 2 cytoskeletal components. Mild fixation of the tissue with glutaraldehyde and formaldehyde prior to ultrathin cryosectioning was the only method that permitted high resolution combined with good preservation of both the polymerized state of microtubules and antigenicity of the antibodies. In addition, these appropriate fixation conditions ensured minimal ultrastructural changes. With light microscopy, fluorescent labelling of ultrathin sections allowed an overview throughout the tissue section of both the cytoskeletal components examined—tubulin and desmin. Since the thickness of the sections was less than the depth of the focus of the objective, high magnification and high resolution images were readily obtained.

The observations made here on immunolabelled sections support most of the previous findings regarding the distribution of intermediate desmin filaments in the areas between the Z disks and near the desmosomes. However, contrary to previous reports concerning other species, we observed that in normal rat heart, as in mouse, there were very few longitudinally oriented desmin filaments and few connections between these filaments and the mitochondria or nuclei. Therefore, in rat heart, the intermediate filaments make up the transverse network that keeps the cellular compartment containing the myofibrillar apparatus in register and might only form interconnections with, and hence exert some positional control over, the mitochondria nearest to the Z disks. Further, Ferrans and Thornell working on hypertrophied human heart and skeletal muscle, respectively, observed an increase in the amount of intermediate filaments, which were either concentrated around the nuclei or packed into the intermyofibrillar spaces, the latter feature often being accompanied by loss of myofibrils. During the onset phase of rat heart hypertrophy studied here, this type of distribution was not observed, even when disorganized sarcomeres were evident. The distribution of desmin filaments bridged the Z disks, whether or not they were in register, and followed the digitations of the intercalated disks. This observation suggests that in the myocytes of heart undergoing hypertrophy, as in skeletal muscle cells, the desmin filaments possibly act as a link that, in conjunction with mechanical forces, aid the alignment of newly formed sarcomeres and keep the myofibrils in register during the contraction-relaxation process.

Our results, together with those of Goldstein and Entman, describe the microtubule distribution as a network mainly forming an irregular lattice of interconnections between cell structures occasionally distributed helicoidally around myofibrils. The whole network was faintly visualized at the ultrastructural level even with the aid of immunogold labelling. Immunofluorescent data emphasized the longitudinal disposition with short transverse segments because of the section thickness relative to the narrow space containing any possible connecting helical profiles.

In normal heart, microtubules constituted the main cytoskeletal structure in the areas close to the mitochondria and to the plasma and nuclear membranes. In nonmuscle cells, membranes specialized in different cell functions were shown to possess high-affinity, large-capacity tubulin binding sites. The present observation that microtubules are located very close to most of the mitochondria is consistent with this finding and tends to support the hypothesis that the position of these organelles in myocytes is partly controlled by the microtubular system. The presence of tubulin along the plasma membrane, together with the findings concerning the role of microtubules in the transport of insulin receptors in adult cardiac myocytes, suggest that microtubules may act as a dynamic transport system in myocytes as in nonmuscle cells.

The increase in fluorescence observed with tubulin antibodies in hypertrophied myocytes on either isolated cells or thin sections were correlated at the electron microscope level with an increase in the number of microtubules in the areas containing nuclei and myofibrils. Our observation of an augmented number of microtubules streaming away from the nuclei towards the sarcoplasm supports previous electron microscope observations in both active hypertrophy and compensatory hypertrophy in rabbit and human.
heart. The presence of large numbers of microtubules in the area around the nuclei, rich in membrane-bound ribosomes and Golgi apparatus, might be related to the role that they were recently reported to play in the processes of protein synthesis and therefore to the transient rise in protein synthesis that occurs during the onset of cardiac hypertrophy.

The increase in the number of microtubules apposed

**FIGURE 6.** In sham-operated rat, desmin labelling is visible in area of intercalated disks, but tubulin labelling is only seen in intermyofibrillar spaces (Panel a, arrows). In hypertrophied rat heart, zig-zag shape of intercalated disk is combined with fairly longitudinal distribution of desmin labelling (Panel b). In other cases, desmin labelling forms longitudinal strands that cover 1 sarcomere and link Z disk to intercalated disk (Panel c). Note absence of tubulin labelling in this area of cell (Panel a: × 25,000; Panels b and c: × 31,250.)
to the myofibrils that were observed in hypertrophied heart may reflect their possible organizational role in the myofibrillogenesis that occurs soon after aortic stenosis. This role might be similar to that described in growing myoblasts or during postpartum growth in rats. However, it also emerges from our data that in hypertrophied myocytes, microtubules remained absent from the Z disks and intercalated disks, both of which are supposed to provide a template for the organization of actin filaments. Connections between actin filaments and microtubules have been reported to form a lattice permitting the aggregation of myosin filaments. Such an aggregation was also observed in the presence of taxol, a polymerizing agent of tubulin, that aligned thick myosin filaments interdigitating with long microtubules constituting pseudo I-bands. The increase in the number of microtubules that were observed along the myofilaments might provide scaffolding for the assembly of myosin filaments in myocytes during the onset of hypertrophy and precede the appearance of the longitudinal desmin filaments associated with out-of-register myofibrils. Indeed, according to studies of skeletal myogenesis, the distribution of desmin filaments at the Z-disks level only occurs after myofibrillogenesis. The completely distinct rearrangement of desmin and tubulin filaments in hypertrophied myocytes emphasizes their probable separate roles in the organization of the cell.

In response to excessive hemodynamic load, myocytes exhibiting ultrastructural alterations were found to be differently distributed throughout the ventricular wall in myocardium developing hypertrophy. The heterogeneous nature of the myocyte response to cardiac overload was confirmed in our previous investigations showing changes in tubulin and isomyosin patterns in a subpopulation of isolated myocytes purified from whole heart ventricles. Here, this observation was extended to papillary muscle considered to be homogeneous.

We conclude that, in myocytes of heart developing hypertrophy, intermediate desmin filaments are chiefly involved in the process of myofibril registration, as previously proposed for chicken skeletal muscle by Tokuyasu et al. Concerning the microtubules, those found along the mitochondrion and plasmalemma are probably involved in cell organelle distribution and in the transport of substances through the sarcoplasm, regardless of the activity of the cell concerned. The fact that, during the onset of cardiac hypertrophy, microtubules increase in number and change in their distribution argues for the possibility that microtubules have a dynamic role in sarcomereogenesis. The present results provide the first in vivo evidence that microtubules polymerize adjacent to the myofibrils when sarcomereogenesis is stimulated in adult heart muscle. Therefore, in striated muscle, the state of assembly of some microtubules is correlated with the activity of the cell and more especially with the process of myofibrillogenesis, whether the muscle cells are in the myoblast stage, the neonatal stage, or the growing adult stage.

Acknowledgments

The authors are grateful to B. Swynghedauw, K. Schwartz, P.Y. Hatt for fruitful discussions, to L.E. Thornell and to D.A. Fischman for constructive criticisms. They are indebted to F. Cluzeau for expert technical assistance, and to M. de Villedon for secretarial assistance.

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KEY WORDS • heart • hypertrophy • microtubules • desmin filaments • myofibrillogenesis
Microtubules and desmin filaments during onset of heart hypertrophy in rat: a double immunoelectron microscope study.

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_Circ Res._ 1987;60:327-336
doi: 10.1161/01.RES.60.3.327

_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1987 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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