Disruption of Microtubules As an Early Sign of Irreversible Ischemic Injury
Immunohistochemical Study of In Situ Canine Hearts

Kunimitsu Iwai, Masatsugu Hori, Akira Kitabatake, Hidetake Kurihara, Kiyohisa Uchida,
Michitoshi Inoue, and Takenobu Kamada

Structural disruption of the cytoskeleton may be involved in irreversible ischemic injury. In the present study, ischemic changes in microtubules during various periods of myocardial ischemia were studied with an immunohistochemical technique in open-chest dogs. In intact myocardium, microtubules were stained as a filamentous network throughout cytoplasm and a circular network around the nucleus, which disappeared with colchicine treatment. In brief ischemia of less than 15 minutes, microtubule patterns were unaltered. After 20 minutes, however, characteristic microtubule stains were partly lost in patchy lesions. As an increase in ischemic period, lesions of loss of microtubule stains were increased in number and size. After 120 minutes of reperfusion following 60 minutes of ischemia, the lesions with intact actin filaments but with disrupted microtubules were replaced by the severely injured cells in which the regular myofibrillar registrations were distinctly disrupted. After 24 hours of reperfusion following 40 minutes of occlusion of the left circumflex artery, the percent area of disrupted microtubules at 40 minutes of ischemia was replaced by that of irreversibly injured lesions in the posterior papillary muscle. These results indicate that disruption of microtubules during ischemia heralds irreversible ischemic injury. However, in in vitro study, the myocardium incubated in hypoxic solution for 60–120 minutes demonstrated earlier disruption of the microtubules than the vinculin. Electron microscopic study also showed minimal irreversible changes in the lesions with disrupted microtubules. Thus, taken together, we conclude that microtubules that support the structural integration of myofibrils and other organelles are disrupted in severe myocardial ischemia before the irreversible injury, promoting the irreversible change after reperfusion. Thus, a loss of microtubule stain could be an early sign of irreversible ischemic injury. (Circulation Research 1990;67:694–706)

The structural deformations of the plasma membranes and disruption of sarcomeres during ischemia are closely related to the irreversible injury of myocardial cells. Although the precise mechanisms underlying these structural defects during ischemia are still unclear, it has been suggested that the disruption of the filamentous network of the cytoskeleton may be involved in irreversible ischemic injury. Steenbergen et al. reported that loss of vinculin in severe myocardial ischemia contributes to detachments of actin from sarcomera, leading to the formation of blebs and rupture of the sarcomemal membranes. The microtubules, which are closely associated with mitochondria, sarcoplasmic reticulum, plasma membranes, and other organelles, may also play an important role in maintenance of structure and function of these organelles. Thus, the disruption of the microtubules during ischemia may be primarily involved in the functional deficiencies of these organelles as well as the structural deformations. Recent immunohistochemical studies in the neuronal cells of gerbils have indicated that the disruption of microtubules during cerebral ischemia are closely related to irreversible injury. In the myocardium, however, histological changes of microtubules during ischemia have not been extensively studied, presumably because of technical limitations.

Recently Kurihara and Uchida developed the immunofluorescence technique that enabled us to stain the microtubules in tissue sections. In the present

From the First Department of Medicine (K.I., M.H., A.K., T.K.) and the Department of Medical Information Science (M.I.), Osaka University Medical School, Osaka, Japan, and Shionogi Research Laboratories (H.K., K.U.), Osaka, Japan. Supported by grant-in-aid 62570392 for scientific research from the Ministry of Education, Science, and Culture, Japan.
Address for reprints: Masatsugu Hori, MD, PhD, The First Department of Medicine, Osaka University Medical School, 1-1-50, Fukushima, Fukushima-ku, Osaka 553, Japan.
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study, we applied this technique to ischemic myocardium in vivo and in vitro and investigated the serial changes of the structure of microtubules as well as those of actin filaments to clarify the role of microtubules in irreversible injury of ischemic myocardium.

Materials and Methods

Changes in Cytoskeleton During Myocardial Ischemia and Reperfusion of In Situ Canine Hearts (Protocol 1)

Experimental protocol. Twenty-nine adult mongrel dogs weighing 14–28 kg were anesthetized with sodium pentobarbital (30 mg/kg i.v.). The trachea was intubated, and each dog was ventilated with oxygenated room air by a respirator to adjust arterial PO$_2$, P CO$_2$ and pH within physiological ranges. The chest was opened through the left fifth intercostal space, and the heart was suspended in a pericardial cradle. The left anterior descending coronary artery (LAD) was cannulated at a site proximal to the bifurcation of the first diagonal branch and was perfused through an extracorporeal bypass tube with blood from the left carotid artery. Arterial pressure was measured at the femoral artery, and regional coronary blood flow was measured by the electromagnetic flowmeter at the bypass tube. The visible epicardial collateral vessels from the left circumflex artery (LCX) were ligated to reduce the collateral flow to the minimum. In a preliminary study, this technique could reduce the collateral flow to less than 5 ml/min/100 g myocardium measured by the time collected blood retrogradely drained to zero pressure at the cannulation site. Regional myocardial ischemia was produced in the LAD area by occlusion of the bypass tube, and dogs were killed at various intervals for histological studies: 15 minutes (n=3), 20 minutes (n=3), 30 minutes (n=4), 60 minutes (n=5), 120 minutes (n=2), and 180 minutes (n=2) after the occlusion. In another three dogs, hearts were reperfused for 120 minutes after 60 minutes of ischemia. Five dogs were used for the control (nonischemic) study, and two were treated with colchicine (1 mg/kg i.v.) 5 hours before they were killed. In the latter seven dogs, hearts were fixed under normoxic conditions. After the completion of the experiment, tissue samples were obtained (see below). Evans blue was injected through the bypass tube to determine the perfusion area. In our series of experiments the coronary blood flow (mean±SEM) before occlusion was 96±2.5 (mean±SEM) ml/min/100 g myocardium.

Double staining of microtubules and actin filaments. Myocardial tissue perfused by the LAD was fixed by perfusion with a periodate-lysine-paraformaldehyde (PLP) fixative at a pressure of 130–180 mm Hg. The perfusion fixation was done in the beating heart. From the center of the LAD area, five transmural tissue samples (5 mm in width) were obtained. They were immersed in PLP fixative for 6 hours and then placed overnight in 4% paraformaldehyde solution (pH 7.4). After fixation the tissue samples were rinsed successively in phosphate buffered saline (PBS) solutions containing 10%, 15%, and 20% sucrose (4 hours each). Cryosections (2 μm thick) were cut using a Tissue-Tek-II microtome/cryostat (Miles Inc., Elkhart, Ind.); then they were mounted on gelatin-coated glass slides and rinsed three times in PBS (30 minutes each). The sections were processed for the double staining of microtubules and actin filaments. The sections were incubated overnight at 4°C with monoclonal antibodies against β-tubulin (Amersham Corp., Buckinghamshire, UK); diluted 1:300 in PBS) and then incubated with fluorescein isothiocyanate–labeled sheep anti-mouse immunoglobulin G (Amersham; diluted 1:30 in PBS) for 1 hour at room temperature. Sections were incubated with rhodamine-phalloidin (Wako, Osaka, Japan; diluted 1:70 in PBS) for 20 minutes at room temperature and rinsed in PBS. All sections were examined by an FX-S RFL fluorescence microscope (Nikon Inc., Tokyo). To test the nonspecific staining, the primary antibody was deleted from the procedures in two control samples.

The extent of the ischemic injury was assessed by the changes in microtubules and actin filaments classified by the following three grades in double immunofluorescence stains: grade 1, both microtubules and actin filaments are normally stained; grade 2, microtubule stains are poor or lost, whereas actin filaments are almost normally stained or cross striations are minimally undulated; and grade 3, microtubules disappear because of distinct disruptions of cross striations or hypercontraction of actin filaments.

Microscopic views (×80) of randomly selected areas from the transmural samples were photographed (40–80 pairs of photographs for each tissue sample), and the lesions of the above three grades were marked by color pens on the photographs. The percent area of the lesions of each grade was obtained by the ratio of the area of lesions to total areas examined.

Electron microscopic examination. For electron microscopic examination of the control and ischemic myocardium, several pieces of tissue samples (1 mm thick) were obtained from the blocks immediately after perfusion with PLP fixative, immersed in 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) at 4°C for 2 hours, fixed in cold phosphate buffered 1% osmium tetroxide for 2 hours, and then dehydrated in graded alcohols. The fixed sample was placed in propylene oxide, placed in a 1:1 mixture of propylene oxide and Epon 812, embedded in Epon 812, and sectioned with a Reichert ultramicrotome. Ultrathin sections were stained with 3% uranyl acetate and 1% lead citrate and examined with a JEOL 100-CX electron microscope (Tokyo).

To study the ultrastructural changes of the myocardium in comparison with the immunohistochemical changes in microtubules, the lesions marked under the fluorescence microscope were fixed with 1% glutaraldehyde for 1 hour and 1% osmium tetroxide for another hour. Then, they were
Immunohistochemical study of vinculin. In another three dogs subjected to 60 minutes of LAD occlusion, the beating hearts were isolated, and five transmural tissue samples obtained from the LAD area were immediately immersed in hexane cooled by dry ice. Nonischemic tissue samples were obtained from the LAD areas of two other dogs as a control. The cryosections were cut (2 μm thick) using a Tissue-Tek II microtome/cryostat and mounted on gelatin-coated glass slides to be fixed with 100% aceton at room temperature for 5 minutes. The slides were dried and rinsed in PBS. For immunohistochemical staining of vinculin, the sections were incubated in monoclonal antibody against vinculin (ICN, Lisle, Ill.; diluted 1:40 in PBS) for 2 hours at room temperature and then incubated with fluorescein isothiocyanate–labeled anti mouse immunoglobulin G (Amersham; diluted 1:30 in PBS) for 1 hour at room temperature. The sections were examined by an FX-S RFL fluorescence microscope. Microscopic views (×80) of randomly selected areas from the transmural samples were photographed, and the lesions of disrupted vinculin were marked. The ratio of the areas of lesions to total areas examined was obtained.

Disruption of Microtubules and Irreversible Changes in Ischemic Myocardium (Protocol 2)

To elucidate the relation between a loss of microtubules and irreversible change of the cells in ischemic myocardium, percent area of microtubule loss in the posterior papillary muscle after 40 minutes of LCX occlusion and percent infarcted area at 24 hours after reperfusion were studied in seven dogs. While the dogs were under pentobarbital anesthesia, their chests were opened and the proximal portion of the LCX was occluded by an umbilical tape after the administration of heparin (100 IU i.v.). In three dogs, after 40 minutes of the coronary occlusion, the LCX was punctured at the distal portion to the occluded site, and PLP solution was perfused to fix the tissue for the histological study. After the perfusion fixation, the heart was excised, and six transmural tissue samples were obtained from the posterior wall, including the posterior papillary muscle. From each tissue sample two cryosections, which included the papillary muscle, were subjected to the immunohistochemical study of microtubules. Percent area of loss of microtubules in the posterior papillary muscles was determined by the point-counting method. The outer margin of papillary muscle was determined by projecting the endocardiac surface of the ventricle. In four other dogs, after 40 minutes of occlusion, the LCX was reperfused, and the chest was closed for recovery from anesthesia. Successful reperfusion was confirmed by the visual inspection of hyperemia in the LCX area, and the recovery of LCX flow was measured by an electromagnetic flowmeter. After 24 hours of reperfusion, the dogs were killed and six transmural tissue samples, including the posterior papillary muscle, were obtained from the posterior wall of each dog as described above. To assess the infarcted area, triphenyltetrazolium chloride staining and modified periodic acid–Schiff staining were performed, respectively. The samples were divided into two transmural pieces. One was incubated in 1% 2,3,5-triphenyltetrazolium chloride (0.1 M phosphate buffer, pH 7.4, 37°C) for 10 minutes, and necrosis was determined as the macroscopically nonstained areas. The other was fixed with 10% buffered neutral formalin. Then, cryosections were made; they were digested with 0.2% α-amylase, followed by routine periodic acid–Schiff staining. In the microscopic view, the necrotic cells showed a strong positive stain. In both stainings, percent area of necrosis was measured by the point-counting method.

In Vitro Study of Disruption of Microtubules and Vinculin During Hypoxia (Protocol 3)

To exclude the effects of collateral flow and mechanical stress on the ischemic changes in the cytoskeleton, the myocardial tissue was subjected to an in vitro hypoxic condition after the modified method of Grochowski et al. In nine open-chest dogs, immediately after the heart was excised, transmural tissue samples were obtained from the center of the LAD area. They were incubated in Krebs' Ringer phosphate solution saturated with O2 gas at 37°C. The transmural tissue samples were divided into three pieces by a freehand technique: subendocardial, midwall, and subepicardial specimens (0.5 mm thick and 3–4 mm in length). The specimens were then put into the hypoxic Krebs' Ringer phosphate solution equilibrated with 95% N2 and 5% CO2 at 37°C (Po2, 28 mm Hg). The solution was continuously gassed for the duration of the incubation. After three different incubation periods at 37°C (i.e., 60, 90, and 120 minutes), the incubation solution was replaced by the hypoxic PLP fixative. After 3 hours of fixation at room temperature the tissue samples were post-fixed with 4% paraformaldehyde buffered with 0.1 M phosphate buffer (pH 7.4) for 4 hours at 4°C. After immersion with 10%, 15%, and 20% sucrose, cryosections were made, and microtubules were stained using the immunohistochemical technique. In two dogs, the transmural tissue samples were also subjected to the study of vinculin with the method described above. For the control study, three tissue samples from each dog were incubated in the oxygenated solution for 120 minutes and then fixed with the same technique described above. Electron microscopic examinations were also performed in one dog for each incubation period with the method described above.

Results

Microtubules in Normal Myocardium

In the normal (control) hearts, microtubule structures were observed as the tortuous filaments loosely organized throughout the cytoplasm, which were...
Figure 1. Double labeling of ventricular myocardium (nonischemic) with mouse monoclonal antibodies against β-tubulin (panels a and c) and rhodamine-phalloidin (panel b). Panels a and b: Longitudinal sections. Panel c: Transverse section. Microtubules stained with the antibodies against β-tubulin form a filamentous network throughout the cytoplasm. Note that the cytoplasmic microtubules are composed of longitudinal and transverse filaments. Part of them forms the circular architecture around the nucleus (panel a). Transverse section shows cross sections of longitudinal microtubules as a number of dots. Fine filaments connecting dots are the transverse filaments of microtubules (panel c). Actin filaments labeled with rhodamine-phalloidin form the cross striations of sarcomeres (panel b). Magnification, x530.
mainly composed of longitudinal and transverse filaments. The longitudinal filaments ran along the longitudinal axis and were thicker and longer than the transverse filaments. Both filaments formed reticulur networks; some of them were localized at the perinuclear space forming a basketlike architecture around the nucleus (Figure 1a). In the cross section of the cell, microtubules were stained as a number of small dots irregularly distributed throughout the cytoplasm (Figure 1c).

These filamentous structures were specific for the microtubules because these characteristic structures disappeared when the anti-tubulin antibody was deleted in the staining procedure. Moreover, administration of colchicine (1 mg/kg body wt i.v.), which depolymerizes the microtubules, markedly diminished the immunoreactivities of the microtubules; the microtubule filaments were finely fragmented into regular, short, hook-shaped fragments with a decrease in density of staining, indicating that the microtubules were depolymerized (Figure 2).

Rhodamine-phalloidin stained the cross striations of the actin filaments in sarcomeres. In the longitudinal sections, the cross striations were regularly arrayed and formed broad bands with an intense fluorescence, which corresponded to the I bands (Figure 1b).

Ischemic Changes in Microtubules in In Situ Hearts (Protocol 1)

Double staining of microtubules and actin filaments.
Fifteen minutes after the occlusion of the coronary artery, no detectable changes were observed in the patterns of the immunoreactivities of microtubules. After 20 minutes of ischemia, however, small patchy lesions appeared and were composed of several myocytes in which the immunoreactivities of microtubules were decreased in both number and intensity (Figure 3). These lesions were distributed in the subendocardial layers in which the cross striations of actin filaments remained almost intact. Thirty minutes after coronary occlusion, patchy lesions with complete loss of microtubule stains were distributed transmurally (Figure 4), whereas the cross striations were almost intact. The size of the lesions ranged between 100 and 240 μm in length in longitudinal sections and between 15 and 60 μm in diameter in cross sections, which were similar to those after 20 minutes of ischemia. The myocytes that lost the cross striations of actin filaments were preferentially located in the subendocardial layer. After 60 minutes
of ischemia, the lesions were increased in both size and number, although the transmural gradient was prominent; in the subendocardial layer, large lesions composed of more than 100 myocytes were observed (Figure 5), whereas in the subepicardial layer, the lesions were much smaller. In the injured cells, the microtubule filaments were irregularly fragmented, distinct from the homogeneous fragmentation after colchicine treatment. The microtubules localized in the peripheral portion of the cytoplasm were prone to disrupt earlier than the perinuclear microtubules. The longitudinal filaments were more resistant to ischemia than the transverse ones. In the cells that completely lost the filamentous structure of microtubules, cross striation of actin filaments were sporadically disrupted. Furthermore, the extent of the disruption varied from cell to cell; in a large number of cells the alignment of cross striations of myofibrils was almost normal or minimally undulated (grade 2 injury [Figures 6a and 6b]), whereas in a small number of cells the intervals of striations were distinctly disrupted (grade 3 injury). In contrast, very few cells with disrupted cross striations and intact microtubules were observed. These results indicate that in the process of ischemic injury, microtubules may disrupt earlier than actin filaments. After 120 and 180 minutes of ischemia, the lesions that lost the microtubule structure were increased in size and fused to become a larger lesion. At 120 minutes the microtubule structures almost disappeared in the subendocardial layer associated with an increase in lesions of grade 3 injury (Figures 7a and 7b).

The heart was reperfused for 120 minutes after 60 minutes of coronary occlusion in another three dogs.
to unmask latent ischemic injury. The staining of actin filaments demonstrated that a number of lesions whose cross striations were severely disrupted (grade 3) were transmurally distributed, and immunoreactivities of microtubules were completely lost in these lesions (Figures 8a and 8b). After 60 minutes of ischemia, the area that showed the loss of microtubules (grade 2 injury) was 36±9% (mean±SEM, n=5) of total area examined, and the percent area of grade 3 injury with severe disruption of actin filaments was 7±1%. In 57±9% of total areas, microtubules were intact, and in these cells the cross striations were also unaffected (grade 1). At 120 minutes after reperfusion, the percent area that showed the intact actin filaments and microtubules (grade 1; 58±4% [n=3]) was not altered. However, the area of grade 3 injury shared 37±4% of the total area after reperfusion, indicating that the areas of grade 2 injury (36±9%) at 60 minutes in which actin filaments were almost intact but microtubules were disrupted were replaced by grade 3 injury after reperfusion. These results indicate that the cells that lost the microtubule stains may have the fragility of sarcomere structures during ischemia and show the severe disruption of regular myofibrillar registrations after reperfusion.

Electron microscopic examination. In 20-minute ischemic myocardium, electron microscopic examination demonstrated patchy lesions composed of several cells with increased intermyofibrillar spaces, indicative of severe intracellular edema, and deformation of the myofibrillar parallel alignment (Figure 9b). Some of these cells demonstrated the dislocation of mitochondria from the intermyofibrillar space to the peripheral space. However, neither bleb formation of the plasma membranes nor amorphous matrix density in mitochondria was observed. In 30-minute ischemic myocardium, patchy lesions, each of which was composed of several cells, showed increased intermyofibrillar space, partial disruption of Z bands, and swelling and disrupted cristae of mitochondria. However, very few cells contained amorphous matrix densities in mitochondria. In 60-minute ischemic myocardium, electron microscopic examination was performed on the cells that had almost intact structures of actin filaments but lost the microtubule network structures (grade 2 injury). The myofibrillar parallel alignments were often deformed; Z lines of the myofibrils were dislocated in the longitudinal

Figure 6. Double labeling of 60-minute ischemic myocardium with mouse monoclonal antibodies against β-tubulin (panel a) and rhodamine-phalloidin (panel b). Note that the cells that have intact microtubule networks and those that completely lost microtubules are clearly separated (panel a). Panel b shows the actin filaments in the same region. The alignment of the cross striations is normal in the cells that have intact microtubules (arrowheads). On the other hand, in most of the cells that completely lost microtubule stains, the cross striations between adjacent myofibrils are minimally undulated (arrows). In a few cells, they show hypercontraction (star). Magnification, ×520.
direction, and some were undulated, resulting in a partial loss of regular myofibrillar registrations. In these lesions there were few cells that had amorphous matrix densities in the mitochondria, indicating irreversible changes. In contrast, in the cells that showed distinct abnormal structure of the cross striations of actin filaments (grade 3 injury), severe disruption of Z band and the amorphous matrix densities in mitochondria were frequently observed.

Disruption of vinculin during ischemia. Immunohistochemistry of vinculin in the longitudinal section of the nonischemic myocardium showed prominent fluorescence at the intercalated disc and punctate stains along the plasma membrane (Figure 10a). After 60 minutes of ischemia, patchy lesions of disruption of vinculin structures appeared, although the severity of the disruption was different among the cells. During ischemia, vinculin located along the sarcolemma was disrupted earlier than the vinculin in the intercalated disc. Disruption of vinculin structures was preferentially distributed in the subendocardial layers (Figure 10b). After 60 minutes of ischemia, percent area of loss of vinculin in the transmural tissue samples was 7±2%, which was much smaller than percent area of the microtubule loss after 60 minutes of ischemia (43±7%).

Disruption of Microtubules and Irreversible Changes After Reperfusion (Protocol 2)

After 40 minutes of LCX occlusion, the posterior papillary muscle showed patchy lesions of disruption of microtubules; the percent area of the microtubule lesions was 41±1% \((n=3)\). After 24 hours of reperfusion following 40 minutes of ischemia, the percent area of the irreversibly injured lesions was similar to that of microtubule loss before reperfusion; percent area of irreversible injury was 34±9\% \((n=4)\) judged by triphenyltetrazolium chloride staining and 42±14\% \((n=4)\) judged by the modified periodic acid-Schiff staining. These results indicate that a loss of microtubule staining at 40 minutes of ischemia reflects a potentially irreversible ischemic injury.

**Microtubule Changes During Hypoxia In Vitro (Protocol 3)**

Myocardial tissue incubated in oxygenated Krebs' Ringer phosphate for 120 minutes demonstrated the intact intracellular filamentous network of microtubules except for a few layers of the cells at the edge of the specimen, which showed a loss of microtubule stain, probably because of damage at excision. In contrast to the oxygenated incubation, the tissues incubated in the hypoxic condition for 60–120 minutes showed the patchy loss of microtubule stains as observed in the ischemic myocardium. The percent area of the loss of microtubule stains was increased as a period of hypoxia was increased; 31±3\% at 60 minutes, 46±6\% at 90 minutes, and 94±10\% at 120 minutes. The transmural gradient was also observed in

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**FIGURE 7.** Double labeling of 120-minute ischemic myocardium with mouse monoclonal antibodies against β-tubulin (panel a) and rhodamine-phalloidin (panel b). Subendocardial myocardium shows almost complete loss of immunoreactivities of microtubules frequently associated with severe disruptions of cross striations (arrows). Magnification, ×350.
the area of lesions at all study intervals; the mean subendocardium/subepicardium ratio of percent area of lesion was 2±1. Electron microscopic examinations of the lesions after 60 minutes of hypoxia showed the intact structures of actin filaments with various degrees of myofibrillar registration abnormalities as observed in the ischemic myocardium in vivo. However, severe disruptions of Z bands and amorphous matrix densities in mitochondria were minimally observed. Examination of vinculin in 60- and 90-minute hypoxic tissues showed much less disruptions than the microtubules (percent area of a loss of vinculin staining; 10±3% at 60 minutes and 17±2% at 90 minutes).

Discussion

The goal of the present study was to delineate the morphological changes in the microtubules in myocardial cells during severe ischemia in vivo and to elucidate the role of microtubules in irreversible ischemic injury. Our observations for this study demonstrated that the disruption of microtubules begins 20 minutes after occlusion of coronary arteries and could be an early sign of irreversible injury.

Technical Considerations

The immunohistochemical technique used in this study has an advantage in delineation of three-dimensional structures of microtubules in the cells, whereas the previous electron microscopic study depicts only the cross-sectional view in an ultrathin section. However, the current immunohistochemical technique could not allow in vivo study and has been applied only to isolated cardiomyocytes. A new technique by Kurihara and Uchida enabled us to stain clearly the microtubules in tissue sections. In our technique, L-lysine in the fixative may have a stabilizing effect on the cytoskeleton. The patterns of the microtubules observed in this study are almost the same as in isolated adult myocytes; the circular patterns around the nucleus and the reticular network throughout the cytoplasm were observed. These structures of microtubules may be neither artificial nor secondary to factors influencing the immunostaining of myocytes, because we used a monoclonal antibody to exclude the factors that may cross-react with proteins other than microtubules. Control staining without the first antibody failed to stain the characteristic filamentous structures, indicating that immunoreactivities were specific to microtubules. Fine fragmentation of the immunoreactive filaments by colchicine treatment also confirmed the specificity of immunoreactivity. However, one may notice a possibility that a loss of microtubule stain is due to inadequate perfusion of the fixative to myocardium.
because perfusion of the fixative after sustained myocardial ischemia may not be uniform in the perfused area, presumably because of the localized no-reflow phenomenon.\textsuperscript{18} If a loss of microtubule stain is caused by a perfusion defect of the fixative, the unstained area of microtubules should be increased after reperfusion because of aggravation of no-reflow after reperfusion.\textsuperscript{19} In our study, however, areas of a loss of microtubule stain were not increased after reperfusion, but areas of disruption of cross striations of actin filaments were increased. Moreover, to estimate the area of perfusion defect, we injected India ink after 60 minutes of ischemia. The defects of perfusion area of India ink were much smaller than the area of microtubule loss in our preliminary study. Furthermore, a patchy loss of microtubule stain was also observed in in vitro hypoxic myocardium, which was independent of perfusion inhomogeneity. These findings indicate that the microtubule loss could not be attributed to inadequate perfusion of the fixative but to ischemic insult.

**Ischemic Changes in Microtubules**

In the present study, no alteration of immunoreactivities of microtubules was observed with ischemia of less than 20 minutes. Initial detectable changes in microtubules were observed 20 minutes after coronary occlusion; the immunoreactivities of the transverse filaments were decreased or lost in patchy lesions, whereas actin filaments appeared unaltered. A loss of characteristic reticular network of microtubules was also observed in the same lesion. These observations indicate that the initial ischemic disruption of microtubules occurs after 20 minutes of ischemia in hearts in vivo.

Ischemic changes of microtubules have not been reported except in a recent report by Ganote et al, who demonstrated the disappearance of microtubules in isolated perfused rat heart subjected to 75 minutes of anoxia (Figure 13 in Reference 5). They observed the change in microtubules by electron microscopic examination in the irreversibly injured cells with subsarcolemmal blebs. Although their findings were compatible with our results, they did not describe the changes in the microtubules in the earlier period than 75 minutes. In our study of in vivo hearts, the initial changes in microtubules were detected at 20 minutes of ischemia.

**Disruption of Microtubules and Cell Injury**

Twenty minutes of ischemia is a critical duration that renders the cell irreversible.\textsuperscript{1,2} Then, one may raise a question whether the disruption of microtubules indicates irreversible injury. To answer this question, we examined the reversibility of cells after reperfusion following 40 minutes of ischemia. The posterior papillary muscles were studied after LCX occlusion because the ischemic changes in the posterior papillary muscle after LCX occlusion are relatively uniform and maximal in the ventricular wall.\textsuperscript{12} After 40 minutes of ischemia, a loss of microtubule stain was observed in 41 ± 1% of the total area of the
posterior papillary muscle. This percent area of microtubule lesion was very close to the percent irreversible lesions judged by triphenyltetrazolium chloride and modified periodic acid–Schiff staining 24 hours after reperfusion. The comparable values between percent area of microtubule disruption at 40 minutes of ischemia and percent area of necrosis in reperfused myocardium strongly suggest that disruption of microtubule structure reflects severe cellular injury destined to be the irreversible change. This is further supported by the fact that the grade 2 injury of microtubules (the microtubules are lost but the actin filaments are almost intact) at 60 minutes after occlusion of the LAD was almost replaced by grade 3 injury (both microtubules and actin filaments are disrupted and ultrastructural irreversible changes are frequently observed) after 120 minutes of reperfusion. On the contrary, the myocytes with intact microtubules were not irreversibly injured. This was supported by the findings that percent areas of myocytes with intact actin filaments and microtubules (grade 1 injury) were not different between before (57±9%) and after (58±4%) reperfusion, and that the ultrastructural signs of irreversible injury, that is, disruptions of plasma membranes and appearance of amorphous matrix densities in mitochondria1,2 were not observed in the myocytes that had intact actin filaments and microtubules.

Although these results indicate that the disruption of microtubules is associated with severe ischemic injury close to irreversible damage, we should be careful to conclude the relation between the disruption of microtubules and irreversible injury. In vitro study of hypoxia demonstrated that 31% of the total area lost the microtubule stains at 60 minutes of ischemia, but the ultrastructural signs of irreversible injury were minimally observed by electron microscopic examination. The disruption of vinculin that may play a key role in irreversible changes was also less than that of microtubules. Therefore, these results indicate that disruptions of microtubules occur before the irreversible change but may be an early sign of irreversible injury.

Role of Microtubules in Irreversible Ischemic Change

Reversibility of microtubule disruption is another important issue. It is clear that the depolymerization of microtubules induced by colchicine is reversible.20 If ATP depression during ischemia enhances depolymerization of microtubules,21 a part of the disrupted microtubules may be recovered after reperfusion. However, this mechanism may work only to a

FIGURE 10. Vinculin staining of the nonischemic ventricular myocardium (panel a) and 60-minute ischemic myocardium (panel b). Immunoreactivities of vinculin show the prominent fluorescence at the intercalated disc and the punctate patterns along the plasma membranes (panel a). After 60 minutes of ischemia, patchy lesions of disruption of vinculin are observed in the subendocardial layers (panel b, arrowheads). Vinculin located along the sarcolemma is frequently disrupted and is disrupted earlier than that along the intercalated disc. Magnification, ×130.
small extent, because the pattern of disrupted microtubules during ischemia was distinct from that of depolymerized microtubules by colchicine (see Figures 2 and 6). Moreover, almost all lesions with disrupted microtubules (grade 2 and 3 injuries) were replaced by severe morphological changes (grade 3), indicating that disruption of microtubules during ischemia may enhance the disruption of cross striation of actin filaments after reperfusion. Indeed, in the preliminary study, we observed that rat cardiomyocytes treated with colchicine are more sensitive to hypoxia than are untreated cells. The microtubules are considered to provide the framework of the cell by their filamentous network and structural supports for the plasma membrane, myofibrils, and other membranous organelles. Thus, disruption of microtubules may largely contribute to the cellular fragility and dislocation of the organelles, which could accelerate the membranous damage of the cell. Vinculin, which is tightly associated with the plasma membrane, may directly support the sarcosomal integrity, and its disruption may result in sarcosomal damage. On the other hand, disruption of microtubules may not directly cause the membranous disruption; however, fragile cells with disrupted microtubules may be easily irreversibly damaged after reperfusion, probably because of the restoration of mechanical stress. Thus, it is not surprising that the myocardial cells whose microtubules are disrupted show irreversible changes after reperfusion, although disruption of microtubules per se does not mean irreversible change. It is most likely that disruption of microtubules promotes irreversible injury and, hence, could be an early marker of cell death.

**Mechanism of Disruption of Microtubules**

The mechanisms underlying the disappearance of the immunohistochemical reactions of microtubules during ischemia are uncertain. However, disassembly of microtubules by increased intracellular calcium, either directly or through calmodulin is a possible mechanism. Oxygen radicals during ischemia may also act as a cause of microtubule disruption because polymerization of the purified tubulin is inhibited by 6-hydroxydopamine (pro-oxidant) in vitro and this reaction is protected by superoxide dismutase or catalase, as observed with other cytoskeletal proteins. Indeed, in the case of oxidant injury of living cells in vitro, cytoskeletal changes were closely related to the disruption of the cell membranes, leading to irreversible injury. The third possible mechanism is a lysis of microtubules by activated Ca-dependent neutral proteases. It has been reported that these enzymes in the skeletal muscle may remove Z lines or α-actinin from the myofibrils through their actions on the cytoskeleton. Although the mechanism underlying the disruption of microtubules during ischemia was not clarified in the present study, we demonstrated that the loss of microtubule stain in ischemic heart could be an early sign of irreversible injury. Further studies are needed to investigate the precise role of microtubules.

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