METHODS

Photochemical Fluorochrome Staining for Detection of Prenecrotic Changes in Cardiomyocyte Myofibrils

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Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 128, No. 9, pp. 356-360, September, 1999
Original article submitted September 6, 1999

Ischemic and metabolic damages to cardiomyocytes induce immediate ultrastructural changes in their myofibrils. Photochemical fluorochromize staining of prenecrotic cardiomyocytes allows to determine with high sensitivity local redistribution of sarcomere mass. It is shown that different types of destructive processes in cardiomyocyte myofibrils underlie similar polarization microscopic picture.

Key Words: *necrobiosis; cardiomyocytes; myofibrils; luminescence and polarization microscopy*

Double refraction of myosin fibrils of sarcomeres allowed to use polarization microscopy for detection of the most early reactions of cardiomyocytes (CMC) to damage [3-6,10,11,15]. These results provide the basis for classification of CMC alterations. We proposed a method of photochemical fluorochromize staining for detection of early necrobiotic changes in CMC based on the formation of luminescent products in CMC after short wave UV (SUV) irradiation [12-14].

Polarized light microscopy of CMC is based on changes in anisotropy and topography of myosin filaments in altered cell. Here we used photochemical fluorochromize staining in polarization microscopy of myofibrils during CMC alterations.

MATERIALS AND METHODS

Occlusion myocardial infarction was modeled in 39 male C57Bl mice weighing 17-20 g. The left carotid artery was ligated at the level of the middle third of the left ventricle under Nembutal narcosis (0.5%, 1.25 ml/100 g body weight). The animals were sacrificed 5 min and 1 day after coronary occlusion. Catecholamine-induced damage to the myocardium was modeled in 34 male Wistar rats weighing 160-270 g. Isopropylnoradrenaline (8-10 mg/100 g) was injected intraperitoneally 0.5-18 h before decapitation.

After decapitation the heart was isolated and incubated at 0°C to termination of contraction and muscle relaxation. The atria were separated and the heart was cut along through the ventricles and septum. The tissue was washed in a cold neutral buffer. Some samples were frozen in liquid nitrogen and 10-μ sections were prepared on an MK-25 cryostate at -18°C, mounted on warm glass slides, dried on air, and exposed to phosphorus pentoxide. Fixed and paraffin-embedded tissue samples were cut into 7-μ sections. Before fluorochromize staining the sections were treated with xylene, alcohol, ammonia, and water to ensure complete removal of paraffin and formaldehyde [2].

Luminescent, polarization, interference, and bright-field microscopy was used in the study. The preparations were stained by the following techniques: hematoxylin and eosin staining combined with Perls reaction, PAS-reaction combined with colloid iron, according to Hale, and hematoxylin orange, PAS-reaction after amylase treatment and hematoxylin-picrofuchsin

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0007-4888/99/0009-0967$22.00

according to Van Gieson combined with resorcin-fuchsin Weigert staining.

Luminescent studies were performed using ML microscopes with an MFE-10 photoextension (for observations and photography) or a point mercury lamp DRSh-100 operated at constant current instead of DRSh-250 lamp and a photometric extension FMEL-1 with a set of probes and diffraction filters for identification of cell areas and measuring their luminescence spectrum. The 365-nm excitation band was cut out with a diffraction filter and VFS-6 glass. Luminescence intensity was recorded on an FEU-79 photomultiplier coupled with a G1B1 recorder. For polarization microscopy Amplival pol.u and Nu microscopes with a photoextension were used. Diffraction microscopic measurements were conducted under a Peraval Interphako microscope by the method of homogenous color field. The scales of substages corresponded to each other for quick adjustment of the examined area of preparation.

Refractive index linearly depends on the concentration not only in solutions but also in condensed substances [16]. This allows us to evaluate the content of substances by the differences in cell pathlength. Determinations, peculiarities, and functional dependencies of diffraction measurements were described elsewhere [1,7]. Cell refraction was determined using normal heptane and monobromonaphthalene (MBN) characterized by extreme refractive indexes (1.338 and 1.665, respectively) as measured on an IRF-23 refractometer at 22°C. SUV illumination was performed with a SVD-120 A mercury lamp (the distance between the source and specimens was about 200-250 mm). Statistical analysis was performed with parametric and nonparametric tests [8,9].

RESULTS

To reveal the mechanism underlying the mosaic pattern of sarcomere luminescence in altered CMC, we determined refractive index and luminescence intensity in some areas of normal (NCMC) and contracted (CCMC) CMC before and after SUV exposure of dry preparations. We also showed that fixation affects characteristics of nonirradiated sections, while water extraction modulates their properties after SUV exposure.

Preliminary observations showed that luminescence spectra of NCMC and CCMC were identical and the used media had no effect on the cell size. The measurements were conducted on CMC cross-sections separately before (20 cells) and after (14 cells) SUV exposure in order to exclude the effect of MBN on the kinetics of the photoprocess. Before and after (180 min) exposure, the intensity of luminescence in heptane and optical pathlength differences in heptane and MBN were recorded. The measurements were repeated 10 min after differentiation of fluorochromize staining unfixed section in water followed by drying.

Luminescence intensity of NCMC and CCMC differed before and after fixation and after SUV exposure before and after water extraction. In all cases, luminescence intensity was significantly higher in cells with contractures than in NCMC (critical significance level below 0.1%). Both fixation and water treatment caused changes in the intensity of NCMC and CCMC luminescence (Fig. 1).

The calculated refractive index remained unchanged for all cells. A correlation between luminescence intensity and optical pathlength differences in heptane was revealed. Specific luminescence intensity (luminescence per unit of pathlength difference) calculated from these data was the same for NCMC and CCMC under all experimental conditions.

Our findings suggest that the increased CCMC luminescence was not due to qualitative photophysical changes in myofibrils of altered cells leading to intense production of luminescent substances during SUV exposure, but due to changes in the concentration of substances in irradiated area.

Necrobiosis of CMC is associated with degradation of its contactile structures manifested morphologically as destruction and sustained pathological contraction of cell myofibrils [3,6]. At the physicochemical level these changes are manifested as dissolution and coagulation of sarcomeres.

Thus, polarization changes in sarcomeres of altered cells can reflect changes in the "ordering", "structural organization" and "total orientation" of the myosin component of sarcomere myofibrils. Photo-

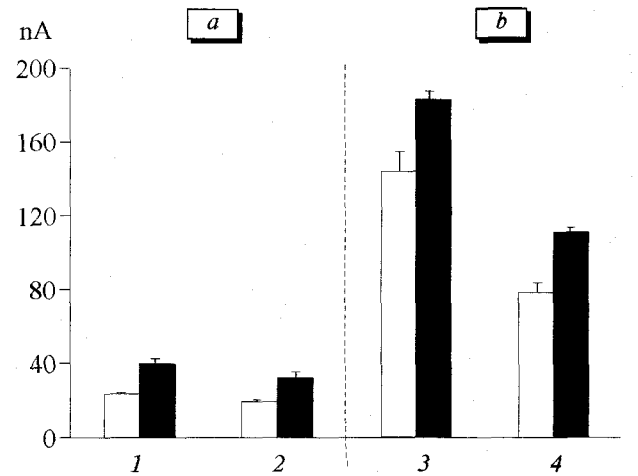

Fig. 1. Luminescence intensity of normal (open bars) and contracted (filled bars) cardiomyocytes during photochemical fluorochromize staining before (*a*) and after (*b*) 180-min short-wave UV irradiation. Before (*1*) and after (*2*) fixation, before (*3*) and after (*4*) water treatment.

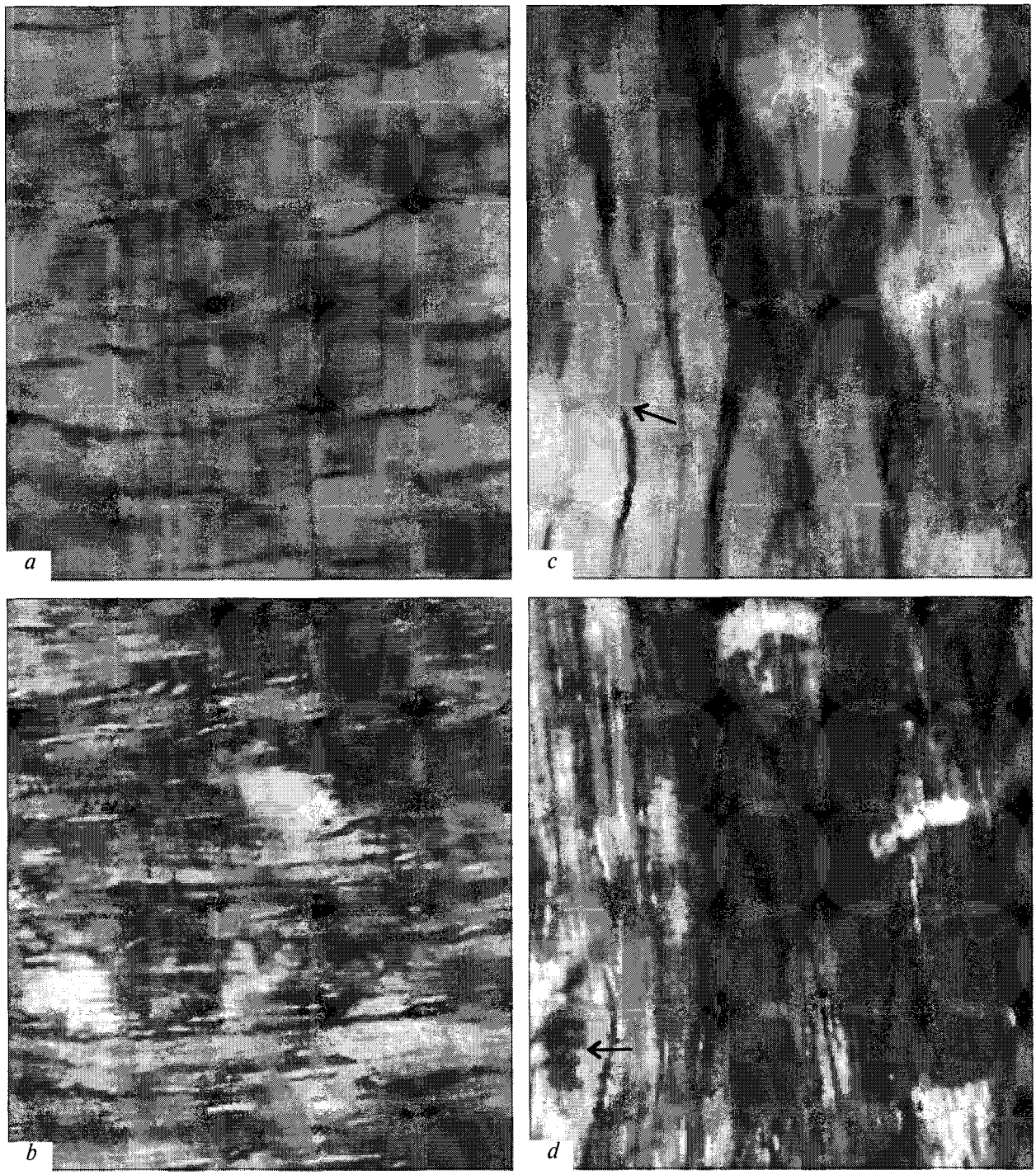

Fig. 2. Prenecrotic changes in cardiomyocyte myofibrils 1 h after isopropylnoradrenaline injection. Cryostate section fixed with neutal formaldehyde and embedded into nonluminescent medium, ×280. *a*) lysed areas with low luminescence intensity adjacent to myofibrils with enhanced luminescence; *b*) the same regions with enhanced and decreased anisotropy; *c*) region of myofibrillar destruction with enhanced luminescence (arrowhead) and contractures with low luminescence intensity; *d*) the same area in polarized light. Luminescent (*a, c*) and polarization (*b, d*) microscopy.

chemical fluorochromize staining reflects redistribution of substances in sarcomeres during necrobiosis.

Luminescent and polarization microscopy revealed 3 types of interaction between the processes of lysis and coagulation during degradation of CMC myofibrils. First type was characterized by mosaic distribution of lysed areas with decreased luminescence and anisotropy and areas with compactly arranged myofibrils with enhanced luminescence (Fig. 2, *a*) and anisotropy (Fig. 2, *b*). In the second type, destruction

of myofibrils in some regions was accompanied by disorganization of their myosin component (Fig. 2, *c*, arrowhead) and anisotropy decrease (Fig. 2, *d*, arrowhead). The weight and luminescence in this region were the same or even exceeded those of contracted regions with enhanced anisotropy. The third type was characterized by contracture-containing regions with highly organized myosin and intense anisotropy but low weight and luminescence intensity (Fig. 2, *c, d*).

Thus, the method of photochemical fluorochromize staining allowed us to reveal ischemic or metabolic prenecrotic changes in CMC detecting with high sensitivity local redistributions of substances in sarcomere compartments. This method revealed a variety of destructive processes of CMC myofibrils corresponding to the same polarization microscopic picture.

REFERENCES

1. G. Beneke, *Introduction in Quantitative Cytochemistry* [in Russian], Moscow (1969), pp. 70-92.
2. M. V. Voino-Yasenetskii and Yu. M. Zhabotinskii, *Errors in Morphological Studies* [in Russian], Leningrad (1970).
3. L. M. Nepomnyashchikh, *Morphogenesis of General Pathological Processes in the Heart* [in Russian], Novosibirsk (1991).
4. L. M. Nepomnyashchikh, *Byull. Eksp. Biol. Med.*, **121**, No. 1, 4-13 (1996).
5. L. M. Nepomnyashchikh, *Alterative Insufficiency of Cardiomyocytes during Metabolic and Ischemic Damages* [in Russian], Moscow (1998).
6. L. A. Semenova and Yu. G. Tsellarius, *Ultrastructure of Cardiomyocytes during Focal Metabolic Lesions* [in Russian], Novosibirsk (1978).
7. G. Tauberg and Kh. Krug, *Iena Review*, No. 5, 218-226 (1972).
8. V. Yu. Urbakh, *Biometricy* [in Russian], Moscow (1964).
9. M. Hollender and D. A. Vulf, *Nonparametric Statistics* [in Russian], Moscow (1983).
10. Yu. G. Tsellarius and L. A. Semenova, *Histopathology of Focal Metabolic Lesions in the Myocardium* [in Russian], Novosibirsk (1972).
11. Yu. G. Tsellarius, L. A. Semenova, and L. M. Nepomnyashchikh, *Focal Lesions and Myocardial Infarction: Light, Polarization, and Electron Microscopy* [in Russian], Novosibirsk (1980).
12. Yu. G. Tsellarius, V. G. Tsimmerman, L. M. Nepomnyashchikh, and L. A. Semenova, *Photochemical Fluorochromize Staining in Pathoanatomical Diagnostics of Prenecrotic Changes and Myocardial Infarction. Method. Recommendations* [in Russian], Moscow (1984).
13. Yu. G. Tsellarius, V. G. Tsimmerman, L. M. Nepomnyashchikh, and L. A. Semenova, *Byull. Sib. Otdelen. Akad. Med. Nauk SSSR*, No. 5, 72-79 (1984).
14. V. G. Tsimmerman and Yu. G. Tsellarius, *Arkh. Patol.*, No. 7, 72-74 (1976).
15. I. D. Shperling, *Ibid.*, No. 9, 62-67 (1987).
16. A. Voehringer and N. Maurer, *Histochemie*, **25**, 234-246 (1971).