Regenerative and Plastic Insufficiency of Cardiomyocytes during Impairment of Protein Synthesis

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Regenerative and plastic insufficiency of cardiomyocytes comprises cell changes resulting from impaired DNA-dependent RNA synthesis and biosynthetic processes. It is accompanied by progressive involution of cytoplasmic structures and causes atrophy and partial elimination of cells without signs of coagulation and colliquative necroses. Segregation and fragmentation of nucleoli in cardiomyocyte nuclei, lysis of myofibrils, and focal degradation of the cytoplasm are ultrastructural signs of impaired protein synthesis. Intracellular cardiomyocyte regeneration is characterized by disorientation of newly formed myofibrils and their excessive elongation, which are related to DNA damages followed by changes in transcription and translation in cardiomyocytes. Calculation of the absolute number of cardiomyocytes is a new approach to evaluation of contractile myocardial insufficiency.

Key Words: *cardiomyopathies; impaired protein synthesis; cardiomyocytes; ultrastructure; population count; non-necrotic death and elimination of cells*

Morphological studies of myocardial pathology revealed two major forms of cardiac insufficiency (alterative and plastic) [8,10-12,41]. Alterative cardiac insufficiency results from acute reversible or irreversible alterations of cardiomyocytes (CM) [9], and its severity depends on the degree of focal ischemic and metabolic myocardial damages [39]. Plastic insufficiency (PI) of the myocardium is characterized by abnormal regeneration of intracellular structures, *i.e.*, disturbed plastic processes in the myocardium, rather than by direct damage to CM [22,28]. These diffuse changes impair contractile properties of CM. This classification was proposed by Russian scientists, who revealed morphological signs typical of these forms of cardiac insufficiency [7,8,11,12,17,38,41]. These studies allow us to evaluate pathogenetic mechanisms of cardiac insufficiency and to develop adequate preventive and therapeutic methods.

The increasing interest in PI of the myocardium is due to the introduction of cytostatics with pronounced cardiotoxic properties into medical practice [38]. These cardiotoxic antineoplastic preparations (rubomycin, adriamycin, doxorubicin, *etc.*) impair cardiospecific gene expression [55]. Studies of the pathogenetic mechanisms underlying PI of the myocardium can provide a clue to the problem of cardiomyopathy (disease of unknown etiology) [18,33].

True PI of the myocardium can be induced by anthracycline antibiotics acting directly on the genetic apparatus of CM. These drugs specifically bind to DNA by intercalating between base pairs in the double helix [68], inhibit DNA-dependent RNA polymerase, and impair intracellular regeneration of CM.

PI of CM is observed during intoxication, total or partial starvation, acute and chronic functional overloads of the myocardium, and congenital diseases [38]. We studied CM PI of various geneses to reveal the general morphological signs of this process. CM were examined during anthracycline-induced damages to the myocardium (single or fractional administration of rubomycin hydrochloride a cardiotoxic dose) [23,35,36], total food deprivation with periods for body weight recovery [13], thiamine and protein deficiency [20,24], alcohol intoxication (ethanol admini-

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stration accompanied by thiamine deficiency) [21], endogenous and exogenous hypercholesterolemia under conditions of experimental atherosclerosis [25], hereditary cardiomyopathy (normotensive W/SSM rats with increased susceptibility to galactose-induced injuries) [19,26], and hereditary myocardial hypertrophy (SHR rats with hereditary spontaneous arterial hypertension) [15,34].

Morphological assays were performed by original methods including stereological analysis of myocardial structures at the tissue and ultrastructural levels [14], electron and polarization microscopies [7], and calculation of the absolute number of CM in ventricular myocardium after alkaline dissociation of fixed tissues (Fig. 1, *a*) [2,3].

Ultrastructural Changes of CM Nuclei and Nucleoli

Structural changes in nucleoli of CM nuclei are the earliest morphological sign of impaired RNA synthesis (Fig. 1, *b*). Ultrastructural dynamic studies of the nucleoli in rat contractile CM over the first 24 h after administration of rubomycin in a cardiotoxic dose revealed the very early changes, which included segregation of their granular and fibrillar components.

It was reported that actinomycin D, specific inhibitor of DNA-dependent RNA synthesis and RNA polymerase activity, causes segregation of nucleoli [63]. The term "segregation" refers to spatial isolation of the fibrillar and granular components in nucleoli [51]. Previous studies showed that inhibitors of RNA and protein synthesis, whose effects are not realized via suppression of DNA-dependent RNA synthesis, do not cause nucleolar segregation [64].

Our previous experiments on rubomycin-treated rats showed that partial segregation of CM nucleoli in some cells started 60 min after treatment and persisted to day 1. This was probably related to temporal heterogeneity of rubomycin incorporation into CM nuclei. It should be emphasized that rubomycin in low concentrations is present in the circulation for 5-7 days [46]. Fragmentation is accompanied by reduction of the granular component, while the fibrillar nucleolonema is degraded into short fragments. The appearance of ring-like nucleoli is the most severe structural damage. Structural changes inducing fragmentation and annulation of nucleoli remain unknown.

Fragmentation and annulation of the nucleoli are less specific signs of impaired DNA matrix properties. These changes are observed after suppression of not only transcription, but also translation [8,13]. Fragmentation of nucleoli in hepatocyte nuclei was found during experimental acute ATP deficiency [61]. It was reported that ATP deficiency inhibits RNA and protein synthesis by the feedback mechanism [65]. Hepatocyte nucleoli undergo fragmentation 2-4 h after administration of methionine and ethionine, which interact with the ATP precursor adenine and cause its deficiency in cells. Therefore, fragmentation of nucleoli during ATP deficiency reflects suppressed energy metabolism in cells [57].

In our experiments, fragmentation and annulation of nucleoli in CM nuclei persisted for 5 days after fractional or single administration of rubomycin in a cardiotoxic dose, which indicated severe primary disturbances in the synthesis of not only ATP and RNA, but also of proteins.

It should be emphasized that heterochromatin is not found in CM nuclei 1 h after administration of anthracycline antibiotics (a result of intercalation). During anthracycline-induced damages, the ultrastructure of CM is characterized by the presence of "active nucleus" and "inactive nucleolus".

Partial or complete blockade of RNA synthesis in the nucleus reduces the content of free ribosomes and polyribosomes in the cytoplasm. Free ribosomes and polyribosomes are absent, and the granular reticulum consisting of short tubules is only occasionally seen in CM with abnormal nucleoli at all terms of observations. Ribosomes reappear only after recovery of normal nucleolus structure.

The very early changes caused by rubomycin also include variations in the amount and morphological structure of CM glycogen. Under normal conditions, human and animal CM contain β-glycogen. Heart diseases are accompanied by the appearance of short branching glycogen chains in CM (small β-glycogen particles) [48].

The amount of glycogen in CM sharply increases in rats receiving rubomycin in a cardiotoxic dose. Electron microscopy revealed sequestration of glycogen 3-4 h after rubomycin administration. Light rings or thin monolayer membranes were found around glycogen agglomerates. The initial increase in the content of glycogen, its sequestration, and autophagy probably reflect suppressed activity or inhibited synthesis of enzymes responsible for glycogen utilization. Glycogen stores become a ballast in cells with impaired synthetic and metabolic processes.

Thus, structural changes in nucleoli and the appearance of branching β-glycogen in cell cytoplasm are general morphological signs of inhibited protein synthesis in cells.

Focal Degradation of Cytoplasmic Organelles in CM

During PI of the myocardium, the cytoplasm of CM contains numerous concentric membranes around the

cytoplasmic matrix and organelles (Fig. 1, *c*). These changes were named as focal degradation of the cytoplasm (FDC) [5]. FDC reflects the reaction of cells to degeneration and necrosis of some subcellular elements. The formation of concentric membranes around damaged cytoplasmic regions (sequestration) leads to the appearance of secondary lysosomes and autophagosomes (autophagolysosomes). FDC in CM was found during epinephrine-induced myocardial injury [7].

Thus, autophagosomes are formed during acute cell alterations. Autophagy plays an important role in the maintenance of homeostasis in organs and tissues: it is responsible for removal of damaged structures, macromolecules, excessive glycogen, and lipids from cells. Focal degradation, partial degradation, and partial necrosis in viable cells were described [1].

FDC and autophagy accompany pathological changes in CM associated with disturbances in protein synthesis. The presence of myelin figures was demonstrated in studies of myocardial ultrastructure in humans and animals receiving cytostatics, whose effects are similar to those of rubomycin [66]. However, the dynamics of this process received little attention.

Our experiments showed that autophagolysosomes are transformed into residual bodies (osmiophilic membrane glomeruli) with degradation of segregated cytoplasmic regions. These multimembrane residual bodies are removed from cells through the sarcolemma and intercalated discs by exocytosis. This is confirmed by their localization in the subsarcolemmal space or near intercalated discs.

FDC in CM is accompanied by accumulation of secondary lysosomes having other composition. These lysosomes are surrounded by monolayer membranes, localized in the peripheral and central regions of CM, and contain osmiophilic and osmiophobic granular components. By the end of observations, these lysosomes are found only in the perinuclear space. Some lysosomes lose their membranes and osmiophilic components; residual bodies appear as osmiophilic granules (lipofuscin).

Partial autophagy of the cytoplasm in CM with diminished or blocked protein synthesis probably reflects cell regression or involution directed to the adjustment of cytoplasm volume to functional state of the nucleus. This assumption is confirmed by the dynamics of ultrastructural changes in CM during starvation and aging [13,15].

Ultrastructural Changes in Contractile Apparatus of CM

PI of the myocardium is accompanied by rapid and pronounced ultrastructural changes in CM myofibrils (Fig. 1, *d*). Lysis of myofibril myofilaments is observed as soon as 3 h after rubomycin administration. Myofibrils become less dense, and narrow spaces appear between A disc myofilaments. Many sarcomeres become loosened. In some cases, all myofilaments within a sarcomere disappear. Myofilaments look like thin bamboo-shaped structures. The distance between the adjacent myofibrils increases. Thinning or even disappearance of myofibrils is most pronounced in the central zone of CM. Fragmentation of Z lines is accompanied by their shift in relation to each other.

CM myofibrils are rapidly renewable structures (myosin half-life is 6-8 days). Therefore, disturbances in biosynthetic processes are rapidly followed by lysis of myofibril. It should be emphasized that energy deficiency in CM can result in total lysis of myofibrils, which is typical of intracellular myocytolysis [9,39, 41,42].

The dynamics and morphological signs of changes in myofibrils during CM PI differ from those observed in intracellular myocytolysis. Intracellular myocytolysis in individual cells is accompanied by intensive lytic processes. By the end of the 2nd day, myofibrils are recovered via intracellular regeneration.

PI of the myocardium is accompanied by diffuse and slow lytic processes, which peak 1-2 days after rubomycin administration. Thinned myofibrils preserve their ultrastructural organization. The T-system of CM is not involved in these processes. In our experiments, polarization microscopy revealed no changes in CM cross-striation during myofibril lysis. It should be emphasized that intracellular myocytolysis was detected for the first time by the absence of anisotropy in considerable cell area. Therefore, changes in CM accompanying attenuated or diminished protein synthesis are not the stage or form of intracellular myocytolysis.

Changes in myofibrils observed during anthracycline-induced cardiomyopathy correspond to diffuse myolysis in acute functional overload of the heart [53]. It is believed that myofibril lysis results from hypoxia or release of lysosomal hydrolytic enzymes [52]. Lysis of myofibrils was also found during diphtheritic intoxication and experimental hypercholesterolemia, *i.e.*, under conditions of suppressed protein synthesis in CM [7,8].

Ultrastructural Changes in CM Mitochondria

Little is known about ultrastructural changes in mitochondria during CM PI. It was reported that these changes include lightening of the matrix and reduction of cristae [45,54,66]; however in the majority of cells mitochondrial structure remains unimpaired.

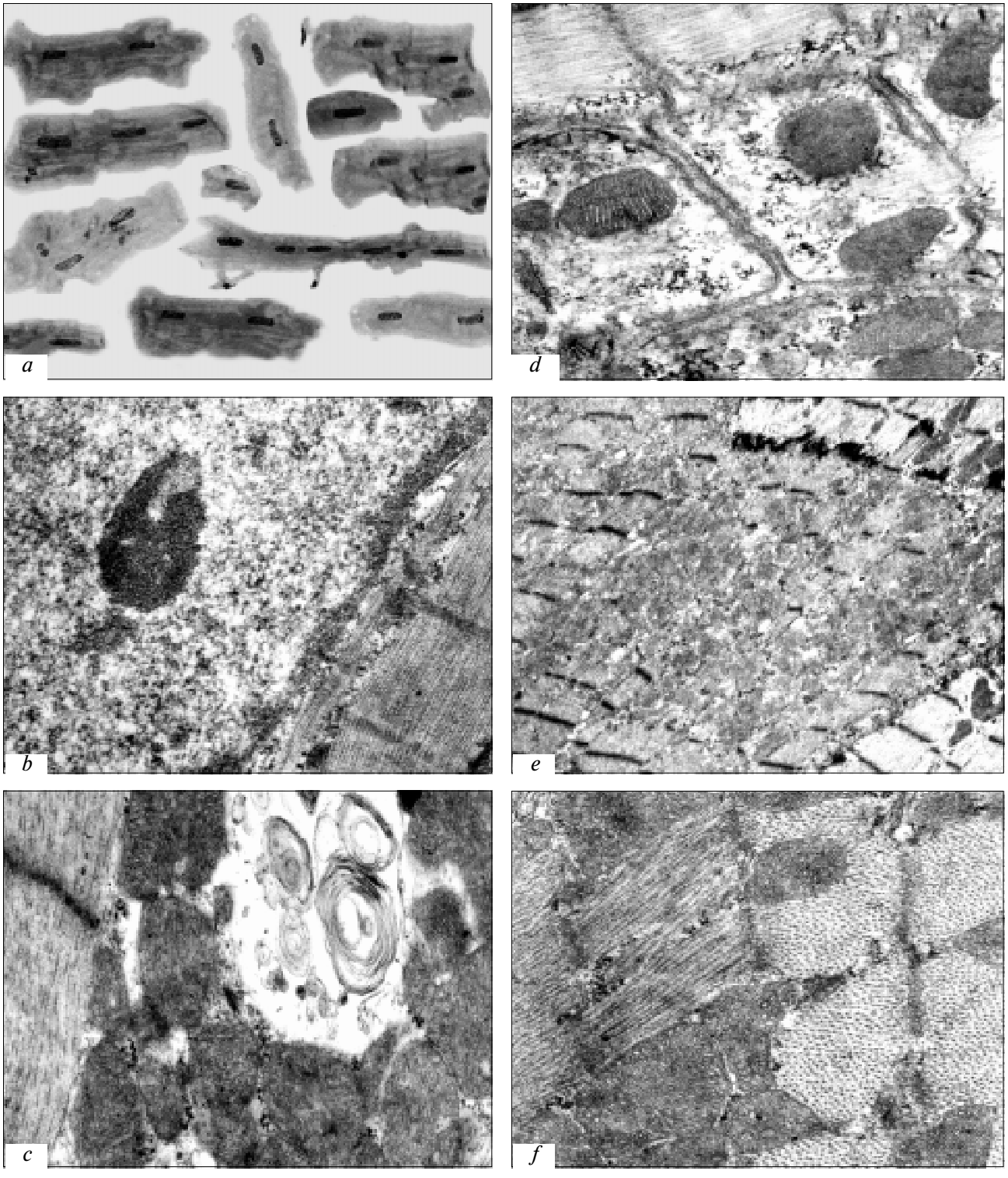

Fig. 1. Ultrastructure of cardiomyocytes during anthracycline-induced cardiomyopathy: isolated cells with various numbers of nuclei (alkaline dissociation, orsein and light green staining, ×1250, *a*); segregation of granular and fibrillar components in nucleoli, decreased density of chromatin lumps (×25,800, *b*); myelin-like bodies and autophagosomes in the cytoplasm, absence of glycogen and ribosomes (×26,000, *c*); lysis of sarcomeres in myofibrils, exposure of T system tubules, small glycogen granules and absence of ribosomes in the cytoplasm (×25,500, *d*); total swelling and destruction of mitochondria, preserved structure of mitochondria in adjacent myocytes (×9700, *e*); and disorientation of myofilaments in myofibrils (×35,200, *f*).

In our experiments, anthracycline-induced cardiomyopathy was accompanied by structural changes in mitochondria. Two days after anthracycline administration, the ultrastructure of mitochondria did not differ from the control. On day 3 after anthracycline administration, individual CM contained enlarged mitochondria with fragmented cristae and lightened matrix. Other ultrastructural parameters did not differ from normal. We concluded that ultrastructural changes in mitochondria reflect intensive cell autolysis.

Static pattern of mitochondrial damages in viable cells suggested that swelling and destruction of cristae occur during sample preparation for electron microscopy and result from destabilization of mitochondrial membrane (Fig. 1, *e*).

Morphological assays did not show whether these changes result from dysfunction of CM nuclei [49,66] or they are related to the effects of anthracycline antibiotics on the genetic apparatus of mitochondria [58]. However, structural analyses indicate that both mechanisms may underlie damages to mitochondria.

If changes in CM mitochondria are related to impaired matrix properties of mitochondrial DNA caused by rubomycin intercalation, damages to mitochondria would appear in a mosaic pattern, mitochondrial population is heterogeneous, and each organelle has its individual cycles of reparation and protein synthesis [28]. Destabilization of mitochondrial membranes in all CM is probably associated with suppressed synthesis of cytoplasmic and structural mitochondrial proteins. Since cytostatics produce different damages to the genetic apparatus of CM, general destabilization of mitochondrial membranes is accompanied by most pronounced inhibition of protein synthesis.

Non-Necrotic Death and Elimination of CM as an Extremely Rare Sign of Myocardial PI

In addition to ultrastructural signs of CM PI, quantitative stereological assays of the total weight of working myocardium and total number of CM illustrates the contribution of these processes into impairment of contractile functions.

Our studies showed that the direct irreversible impairment of transcriptional and translational mechanisms leads to rapid CM death. Light and electron microscopy failed to reveal this process in the myocardium, because cell death was not accompanied by the development of coagulation or colliquative necrosis. It was shown that cell death during myocardial PI is similar to programmed cell death (apoptosis). This process was specified as "CM disappearance" [37], since the mechanism underlying elimination of contractile CM is poorly understood.

As differentiated from cell necrosis, apoptosis and CM disappearance do not lead to the formation of leukocyte infiltrates and focal sclerosis. Calculation of the total number of ventricular CM showed that the heart loses 30% cells over a short period without visible signs of structural changes. Plastic disturbances in the myocardium produce irreversible contractile cardiac insufficiency after the loss of not less than one third of the total weight of CM.

Measurements of cell count and DNA content in the myocardium of patients treated with various cytostatics (cyclophosphamide, vincristine, purinetol, and methotrexate) demonstrated a decrease in the number of CM in children, while in adults this parameter did not differ from the control [43]. Cytostatics, whose mechanisms of action differed from those of daunomycin, had no effect on CM ploidy in children and adults. In one child treated with cytostatics and daunomycin, CM nuclei were primarily diploid, but not tetraploid, as could be expected from the age of this patient and the weight of the myocardium.

Elimination of CM with nuclei of different ploidy remains unknown. A sharp decrease in the number of polyploid nuclei in the myocardium of humans and rats treated with daunomycin probably results from disappearance of CM with tetraploid nuclei, but not from attenuated DNA synthesis in cell nuclei.

Our experiments demonstrated elimination of cells with various numbers of nuclei as follows from the constant ratio between mono-, bi-, and trinucleate CM in the myocardium of control and treated animals under various experimental conditions.

The question arises: which pathological processes and mechanisms underlie the phenomenon of CM disappearance?

Accelerated lysis and autophagy of intracellular structures followed by resorption of residual bodies and autophagosomes by macrophages most probably underlie CM disappearance. It should be emphasized that programmed cell restoration in normal tissues, partial elimination of cells during the early morphogenesis, and metamorphosis are realized via the same mechanisms [44].

Programmed cell death depends on *de novo* protein and mRNA synthesis [4]. These processes are accompanied by internucleosomal cleavage of DNA by endogenous Ca^{2+}- and Mg^{2+}-dependent endonucleases. In an electric field, oligonucleosomal fragments are separated in a staircase manner (by their molecular weights). The staircase type of cell death was named apoptosis [4]. Cytological signs of apoptosis include condensation of the cytoplasm and nuclear heterochromatin, formation of apoptotic bodies and membrane vesicles, and marked reduction of cell volume [59]. Programmed cell death may proceed without

DNA fragmentation (non-staircase type) [47] and morphological signs of apoptosis [60]. Processes accompanied by the appearance of morphological signs of apoptosis and internucleosomal fragmentation of DNA without *de novo* synthesis of mRNA and protein are not considered as programmed cell death [62].

Changes in pre-early gene expression induced by endogenous and exogenous factors and impairing reprogramming of late gene activity constitute the molecular and genetic mechanisms underlying apoptosis during proliferation and differentiation. These processes destabilize DNA and chromatin, increase genetic instability, and trigger programmed cell death [4].

Ultrastructural signs of impaired biosynthetic processes, as well as cytological changes typical of apoptosis (reduction of cell volume), were observed in experimental CM PI. Atrophic changes in CM (involution) accompanying regenerative and plastic insufficiency are related to self-organization of supramolecular structures and macromolecules (programmed process). The final stage of morphofunctional reconstruction in CM resembles apoptosis. As differentiated from apoptosis, atrophic changes in CM are partially reversible. Such morphofunctional changes in cells could be named apoptotic state: these cells can undergo both restoration and non-necrotic death.

Peculiarities of Intracellular Regeneration of CM

CM PI is reversible in the case of early recovery of protein synthesis. However, some CM lose the ability to regenerate after long-lasting and profound changes in mRNA synthesis or translation. Morphological signs of intracellular regeneration of CM (formation of new myofilaments on polyribosomes and normalization of the structure and number of myofibrils) are observed after the appearance of ribosomes in their cytoplasm [40].

The peculiarity of intracellular regeneration of CM during anthracycline-induced cardiomyopathy in SHR rats was disorientation of newly formed myo-

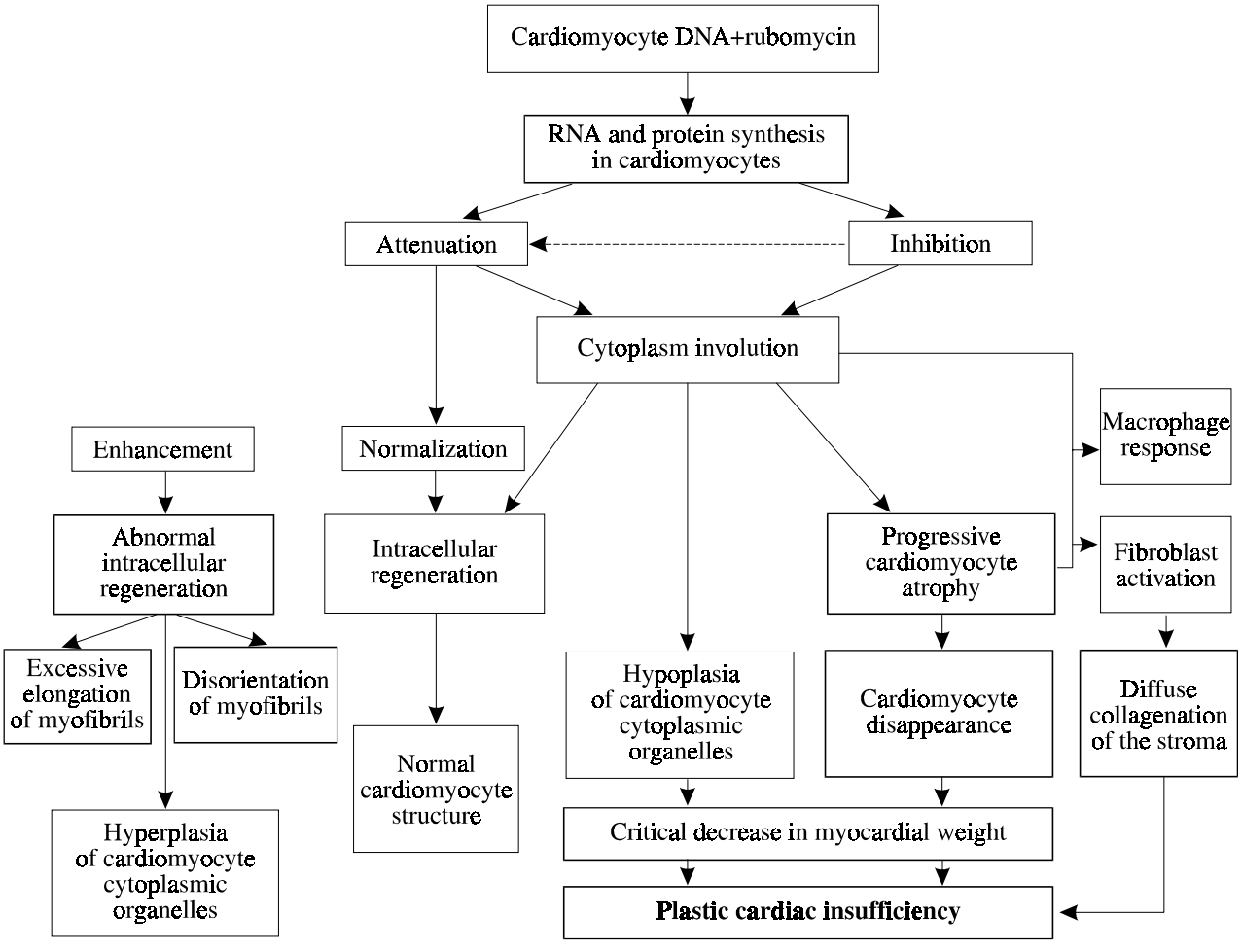

Fig. 2. Development of regenerative and plastic cardiomyocyte insufficiency. Dynamics of dose-dependent changes in myocardial parenchyma and stroma during impaired synthesis of contractile proteins in cardiomyocytes during anthracycline-induced cardiomyopathy.

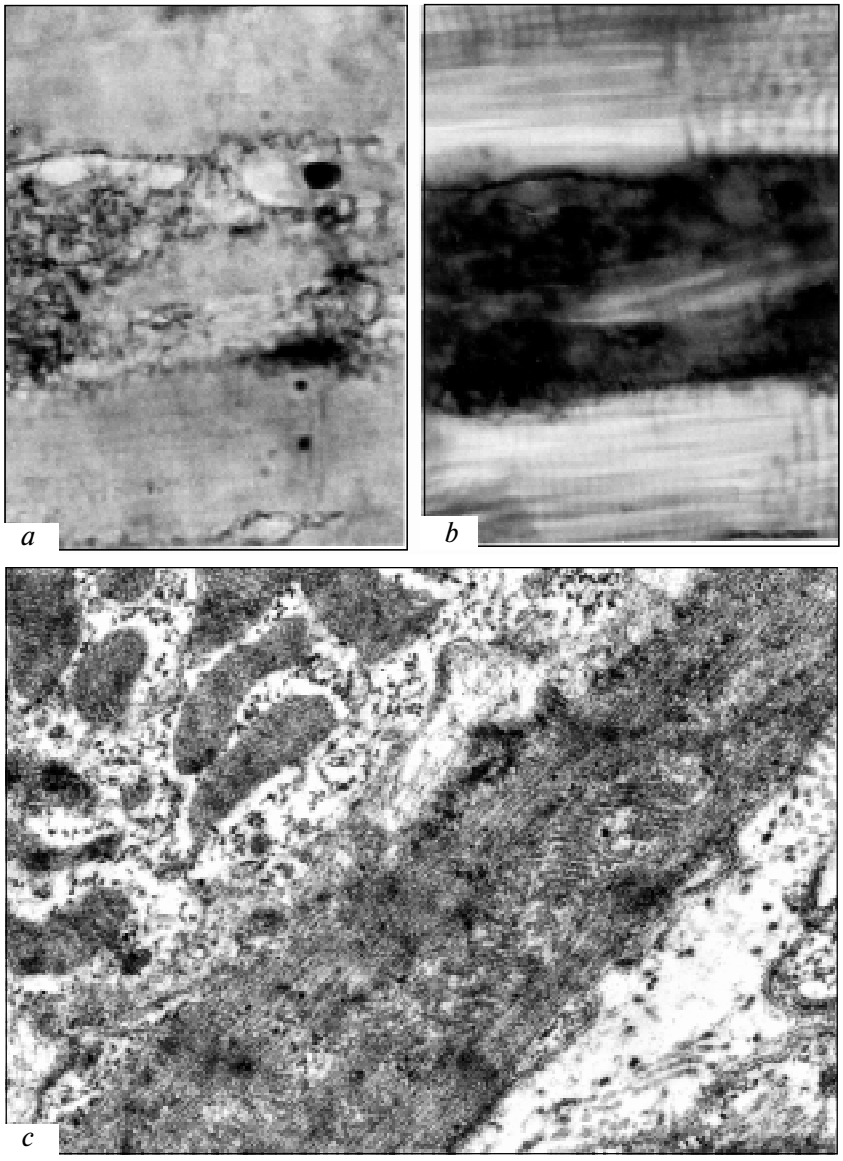

Fig. 3. Atrophy of cardiomyocytes during anthracycline-induced cardiomyopathy: atrophic muscle fiber surrounded by glycosaminoglycan agglomerates (colloidal iron-PAS reaction-hematoxylin, ×800, *a*); the same muscle fiber in polarized light (×27,300, *b*); and high density of preserved myofibrils in atrophic myocyte (×23,900, *c*).

fibrils (Fig. 1, *f*). In the subsarcolemmal and perinuclear spaces and between preserved myofibrils, the newly formed myofibrils were oriented perpendicular or at angle to the long axis of muscle fibers, or extended from the Z line (in a fan-like manner) and intersected with each other. Disoriented myofibrils were also found within individual or CM clusters.

Abnormal regeneration of myofibrils is probably related to DNA matrix damages. Probably, impaired regulatory mechanisms are not normalized even after restoration of RNA-synthesizing properties of nucleoli.

Our experiments first demonstrated cytostatic-induced disorientation and excessive elongation of myofibrils in CM. At the same time, disorientation of myofibrils was shown in studies of ultrastructural mechanisms underlying idiopathic myocardial hypertrophy in humans [56]. This process probably contributes to the geometric integrity of cells and prevents CM enlargement. The constant diameter of muscle fibers in the myocardium is probably under genetic control.

Morphogenesis of Myocardial PI during Impairment of DNA-Dependent RNA Synthesis in CM

Figure 2 shows changes in cells and tissues that result from impaired DNA-dependent RNA synthesis in animal CM. This scheme illustrates the major pathogenetic stages of contractile cardiac insufficiency. Morphological reconstructions in the myocardium agree with the dynamics of changes in the protein-synthesizing system during adriamycin-induced cardiomyopathy [67].

We hypothesize that spatial and temporal heterogeneity of involutive and regenerative changes in CM is due to different number of rubomycin molecules incorporated into nuclear DNA. There is only slight

and short-term inhibition of RNA and protein synthesis in some CM, while in others this long-lasting process causes hypoplasia of intracellular elements. RNA and protein synthesis is completely blocked in some CM, which leads to progressive cell atrophy and elimination.

Physiological heterogeneity of CM due to individual cycles of renewal of intracellular structures probably underlies asynchronous involvement of various CM into pathological processes [6-8,11,16,27-32,39].

The number of CM with severe disturbances in DNA-dependent RNA synthesis increases with increasing a single dose of preparations. The effects of anthracycline antibiotics on cells are similar to those of actinomycin D, whose influence depends on permeability of cell membranes (*i.e.*, total RNA and protein synthesis in organ or tissue cells) [50].

Biochemical and morphological assays showed that the initial inhibition of RNA and protein synthesis is followed by its marked activation, which leads to hyperplasia of intracellular structures (regenerative hyperplasia).

Light and electron microscopy revealed irregular thinning of myocardial fibers by the end of observations (Fig. 3). Activation of contractile protein synthesis also manifested in excessive elongation of myofibrils on days 3-4 of the experiment. It should be emphasized that elongation and wrinkling of myofibrils coincided with most intensive elimination of CM. These processes are probably closely interrelated: abnormal muscle fibers in lysed CM are progressively substituted by adjacent elongated cells.

Figure 2 shows the dependence of changes in the myocardial stroma on functional and metabolic state of the heart parenchyma with impaired synthesis of contractile proteins during anthracycline-induced cardiomyopathy.

Conclusion

Many questions remain to be solved. This review was designed to bring to the attention of scientists the morphological aspect of regenerative and plastic insufficiency of CM, which plays an important role in the pathogenesis of impaired contractile functions.

Thus, the impairment of protein synthesis under cytopathic effects of cardiotropic factors underlies regenerative and plastic insufficiency of CM. Disturbed regeneration of cell ultrastructures decreases the total number of CM due to partial atrophy and elimination of cells without signs of coagulation or colliquative necrosis. Calculation of the absolute number of CM is a new approach to the problem of cardiac insufficiency. This method would allow us to substantiate individual differences in the adaptation to functional overload and to estimate myocardial insufficiency threshold.

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