Endomitosis and polyploidization of myocardial cells in the periphery of human acute myocardial infarction

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Abstract

Objective: Although the genetic program for reinitiating DNA synthesis exists in post-mitotic cardiomyocytes, and it was reported that in human acute myocardial infarction (AMI) a significant proportion of myocytes enter mitosis, the rule is that the lost tissue is replaced by a collagen scar. The purpose of this study was to search for the basis of this discordance in order to devise future strategies to induce division of myocytes into daughter cells that may replace the lost tissue with contractile cells.

Methods: In 15 human hearts with 1- to 21-day-old infarcts, the expression of the cell cycle proteins Ki67 antigen, cyclins D, A, and B1, the presence of mitotic bodies, and the ploidy status were investigated with immunoenzymatic methods, light and laser confocal microscopy, and densitometry in the myocytes surrounding the infarct area.

Results: In 7- to 13-day-old infarcts, 11.61 ± 6.94% of the myocytes presented Ki67+ nuclei, and a lower proportion presented cyclins D, A, and B. At earlier and later times, the proportion of Ki67+ myocytes was significantly lower. Although under confocal microscopy and fluorescent labels, some of the Ki67+ myocytes appeared to be in different stages of mitosis, with Nomarski optics and hematoxylin counterstaining, the condensed chromosomes, although arranged in metaphase and anaphase plates or split in sister chromatids, were always located within a preserved nuclear envelope, indicating the presence of endomitosis. Conventional mitosis was exceptionally observed. In the 14- and 21-day-old infarcts, the ploidy of the myocytes adjacent to the infarct was significantly higher than in distant zones.

Conclusion: These observations indicate that in human infarcts, entrance of cardiomyocytes into the cell cycle is transient and that endomitosis, leading to polyploidy, rather than mitosis, leading to karyokinesis, is the final fate of cycling cells. Both observations may account for the discordance between the regenerative ability of myocytes and the lack of an efficient reparative process in human AMI.

Keywords: Cell differentiation; Hypertrophy; Infarction; Myocytes; Remodeling

1. Introduction

In the last few years convincing evidence has been published showing that the genetic program for reinitiating DNA synthesis exists in post-mitotic cardiomyocytes [1], and that in human acute myocardial infarction (AMI) a proportion of adult myocytes enter in mitosis and proceed towards cytokinesis with formation of daughter cells [2]. However,
the rule is that in acute myocardial infarction the lost tissue is replaced by a fibrous scar, and subsequent pathological remodeling is one of the leading causes of heart failure [3,4]. This is the main reason for the increasing number of studies attempting to replace the missing cardiomyocytes with skeletal myoblasts or progenitor stem cells [5,6].

The mechanisms underlying the discordance between a putative myocyte replication ability and the lack of an efficient reparation are poorly understood. At present it is not known if the newly formed cells have low viability and die after completion of mitosis, if they enter in a quiescent G0 state after a few replication cycles or if the time of myocyte replication is very brief and the number of newly formed cells is negligible as compared with the whole myocardial mass. In addition, since it is known that in the human heart the cardiomyocytes surrounding fibrous scars display a high degree of polyplodization and hypertrophy [7,8], the possibility that the final result of the mitotic activity is the formation of polyploid hypertrophic myocytes should also be considered.

With the aim of exploring which of these mechanisms could be involved in this deficient reparative process, we studied human hearts collected between 1 and 21 days after diagnosis of an AMI.

In this report we present evidence that in human AMI entrance of the myocytes into the cell cycle is transient and that in these cells endomitosis, leading to polyploidy, instead of conventional mitosis, leading to karyokinesis, is the main mechanism of chromosome duplication. Both observations may account for the discordance between the regenerative ability of myocytes and the lack of an efficient reparative process in human AMI.

2. Methods

2.1. Patients

The hearts of 15 patients with left ventricle AMI were obtained. Nine were collected after necropsies performed within 7 h after death and six were hearts explanted from transplanted patients.

Time of evolution of the condition was determined since diagnosis, defined as CK elevation twice the normal value or CK-MB > 24 UI/ml, plus ST-segment elevation of > 5 mV in two or more consecutive leads. In no case the time elapsed between onset of symptoms and diagnosis exceeded 8 h. Table 1 shows the patient characteristics and time of evolution. The investigation conforms with the principles outlined in the Declaration of Helsinki.

As controls we employed 5 human hearts that were rejected for heart transplantation because of unsuitability of the donor (n = 3) or excessive time from organ collection. Donors were 17–34 years old and three were men. Heart weights ranged between 175 and 305 g, and no gross or microscopic abnormalities were detected.
2.2. Tissue preparation

The left ventricle was sectioned normally to the longer axis in 1-cm-thick slices that were fixed for 72 h in 10% phosphate buffered formaldehyde, divided in fragments and embedded in paraffin. Four micrometer thickness tissue sections were stained with hematoxylin–eosin, Gomori’s trichrome and Feulgen’s.

2.3. Immunohistochemistry

The tissue sections were deparaffinized and brought to PBS, pH 7.2. After blocking endogenous peroxidase with 3% H2O2 in methanol and antigen retrieval pretreatment with citrate buffer in a microwave oven, the slides were incubated 1 h with monoclonal antibodies against the Ki67 antigen and cyclins D1, A and B1 (Novoceastra Laboratories, Newcastle upon Tyne), anti-CD68 (Dako, Carpinteria, CA, USA) and anti-smooth muscle actin (Biogenex, San Ramon, CA, USA) and post-treated with a biotinylated anti-mouse immunoglobulin antiserum (Biogenex), followed by peroxidase-labeled avidin and revealed with AEC as chromogen. Subsequently, the sections were incubated with an anti-sarcomeric α-actin antibody (Novoceastra), post-treated with the biotinylated antiserum followed by alkaline phosphatase-labeled streptavidin (Biogenex) and Fast Red as chromogen, and counterstained with hematoxylin. Alternatively, after incubation with the anti-Ki67 and biotinylated antibodies, sections were treated with fluorescein-labeled streptavidin (Vector Laboratories, Peterborough, UK), followed by incubation with rhodamine-labeled phalloidin (Sigma, St. Louis, MO). The tissue sections treated with enzyme-labeled avidin were examined under Nomarski optics or conventional light microscopy, and those with the fluorescent reactants were examined with a confocal microscope (Zeiss, Oberkochen, Germany). Specificity tests, performed by omission of the specific antibody and incubation with non-immune mouse serum, produced negative results.  

The determination of the proportion of cardiomyocyte nuclei expressing cell cycle proteins was carried out in those located in the three cell rows immediately adjacent to the infarct area. The mean number of examined myocyte nuclei in each heart ranged between 846 and 1138.

In order to control the accuracy of the Ki67 stain for identification of cycling cells, tissue sections of two of the hearts with the highest number of Ki67 positive myocytes were incubated with a monoclonal antibody against phosphorylated histone H3 (Sigma), an additional marker for cell division [9], and processed for immunoenzymatic study.

2.4. Determination of ploidy

For each case the nuclear DNA concentration of 200 cardiomyocytes located at the infarct border and 200 located in distant areas was determined in Feulgen-stained tissue sections with the aid of a digital analysis system (Vidas Kontron, Zeiss, Germany). For assessing the accuracy of measurements on tissue sections, myocytes were isolated from small samples of the left ventricle free wall of two control hearts by means of collagenase digestion with a method adapted for human samples [10]. Isolated cells were fixed in suspension in 4% buffered formaldehyde, washed in PBS, dried on Vectabond (Vector) coated glass slides and stained with Feulgen. The data of the nuclear DNA content of these cells were compared with the DNA content of myocyte nuclei examined in Feulgen-stained tissue sections from the same hearts. For reference cells, the nuclear DNA content of non-myocyte cells in the same tissue section, or contaminating the smears of collagenase-isolated cells, that were assumed to be diploid, was determined.

2.5. Statistics

Variables were expressed as mean ± S.D. The statistical significance of differences between groups was determined by one-way analysis of variance (ANOVA) and Scheffe’s test as appropriate. P values <0.05 were considered statistically significant. The data were analysed using the SPSS® 6.1 statistical analysis software for Windows (SPSS Inc).

3. Results

3.1. Patients and infarct pathology

The mean age was 53.5 ± 15.6 years, range 19–72 years. Eleven were male and four female. In two young female patients with Marfan syndrome infarction was due to dissection of the left anterior descendent coronary artery. In all cases the infarcts involved extensive areas of the left ventricle and their histological patterns corresponded grossly with the time of evolution. In three hearts collected at about 1 week after the onset of AMI, the infarct area was invaded by inflammatory cells, mainly CD68-positive macrophages. In recent infarcts (less than 48 h evolution) no inflammatory reaction was seen, with the exception of a peripheral infiltration of polymorphonuclear leukocytes, and in the older infarcts, collected between 12 and 19 days after diagnosis, smooth muscle actin-positive cells, interpreted as proliferating myofibroblasts, were observed at the periphery of the infarct area. Both the CD68 and the smooth muscle actin positive cells displayed Ki67+ nuclei, mitotic activity and occasional cytokinesis.

Tissue preservation and staining with immunologic reactants was similar in hearts collected after necropsy or after transplant. This could be ascribed to the short time elapsed between collection of the material and fixation (less than 7 h in the former and less than 60 min in cardiac explants).
3.2. Expression of cell cycle proteins and cardiomyocyte mitosis

In some of the hearts with MI we observed numerous myocytes, identified by the presence of sarcomeric α-actin in their cytoplasm, their shape, size (more than 15 μm in transversal diameter at the nuclear level) and presence of sarcomeres, expressing Ki67 antigen in their nuclei (Fig. 1, panel A). The pattern of Ki67 antigen stain was variable. Usually it was diffuse, but at times rounded structures of variable size could be observed (Fig. 1, panel B).

On account that almost all the Ki67+ myocytes were dispersed in the two or three rows of surviving myocardium immediately adjacent to the infarct area, and were rarely observed in distant zones, we estimated their proportion only in the periinfarct zone.

As can be seen in Table 1 and in Fig. 2A, the highest proportion of periinfarct myocytes expressing the Ki67 antigen was observed at the second week after diagnosis (11.61 ± 6.94%), and was significantly higher (P = 0.004) than in the hearts collected at earlier (0.334 ± 0.75%) or later times (2.66 ± 3.04%).

In tissue sections consecutive to those employed for search of the Ki67 antigen, myocytes with similar characteristics and location were positive for markers of different stages of the cell cycle, cyclin D (Panel E), cyclin A (Panel

![Fig. 1. Ki67 and cyclin labeling of myocytes in an infarcted heart. In panel A, a low power view of the border of an 8-day-old infarct can be seen. Immediately adjacent to the inflammatory infiltrate (asterisks) three myocytes (cytoplasm stained red after demonstration of sarcomeric α-actin) display Ki67+ nuclei (arrows). Many of the non-myocyte cells present in the infarct area also show Ki67 positive nuclei (arrowheads). Panels B to E show cardiomyocyte nuclei positive for the Ki67 antigen (panel B), phosphorylated histone-3 (panel C), cyclin A (panel D) and cyclin D1 (panel E). Panel F documents myocytes positive for cyclin B1, either cytoplasmic (arrowheads) or translocated to the nucleus (arrow). Sarcomeric α-actin was not investigated in panels D, E and F in order to detect the cytoplasmic presence of cyclins. Panels G and H show that myocytes in M phase of the cell cycle present different appearances according to the method employed. The image in panel G, obtained with a confocal microscope and fluorescent labels, shows what can be considered a metaphase, with advanced chromosome spiralization (arrow). Red fluorescence shows staining of myocyte cytoplasm by phalloidin, and green fluorescence shows chromosomes labeled with the anti-Ki67 antibody. Panel H shows two myocytes examined under Nomarski optics and immunoenzymatic methods. The red-stained cytoplasm reveals the presence of sarcomeric α-actin and the condensed Ki67+ chromosomes are contained within the boundary or a preserved nuclear envelope (arrows). The nucleoplasm appears blue after hematoxylin stain. Panels I and J illustrate myocytes displaying a transversally sectioned metaphase plate (panel I) and two telophase plates (panel J). In both cells the cytoplasm is stained red with the anti-α-sarcomeric actin antibody and the Ki67+ chromosomes plates (arrows) are located within a blue stained nucleus. Panel K shows a myocyte (cytoplasm positive for sarcomeric α-actin) with a conventional mitosis in early anaphase, with start of separation of the Ki67+ chromosomes (arrows).]
D) and cyclin B1 (Panel F). In the hearts collected at earlier and later times, the number of cardiomyocyte nuclei positive for cell cycle proteins was lower or nil (Table 1).

In two hearts collected at the second week after diagnosis we observed numerous myocytes located at the infarct border with their nuclei stained with the anti-phosphorylated histone H3 antibody. The staining pattern was similar to that observed with the anti-Ki67 antigen antibody, and condensed chromosomes were seen within the boundary of an intact nuclear envelope (Fig. 1, Panel C). The proportion of myocytes with positive phosphorylated histone H3 nuclei was lower than the Ki67 antigen-positive cells, 9.33% in patient 7 and 14.22% in patient 8.

Despite a thorough search, we encountered only three classical mitotic bodies in two of the examined hearts, one in an explanted heart collected 7 days after the acute episode (Fig. 1, panel K), and two in a heart from a necropsy performed 12 days after the acute episode. However, in hearts collected between 7 and 12 days after diagnosis, we observed myocyte nuclei with condensed Ki67+ chromosomes, occasionally spliced in sister chromatids, dispersed within the nucleus or arranged in structures similar to a metaphase plate (Panel I), or disposed in clumps located at opposite poles of the nucleus, resembling late anaphase or telophase (Panel J) of conventional mitosis, always within the boundary of an intact nuclear envelope (Fig. 1, panel H). As occurred with the Ki67+ myocytes, the number of endomitosis, although lower, peaked in the hearts collected at 7–8 days after infarction. At earlier and later times endomitosis were exceptionally observed (Table 1).

When tissue sections consecutive to those observed with Nomarski optics and immunoenzymatic methods were examined after processing with fluorescent labels and under laser confocal microscopy, numerous cells with phalloidin stained cytoplasm and containing Ki67+ chromosomes arranged in metaphase or anaphase plates were observed (Fig. 1, panel G). Since with this approach the nuclear envelope could not be identified, we could not determine if chromosomes were disposed within that structure.

In the five control hearts no mitotic figures were observed with both microscopic approaches, and the proportion of Ki67+ myocyte nuclei was 0.43 ± 0.22%.

3.3. Ploidy status of cardiomyocyte nuclei

As can be seen in Table 1, although there was a wide variation in the ploidy status for each patient, in the samples collected at the third week after diagnosis, the difference between the proportion of octaploid myocytes in the neighborhood of the infarct and in the remote areas was significantly higher (P = 0.02) than in the hearts collected at earlier times (Fig. 2, panel B).

In the five control hearts 21.93 ± 6.62 (S.D.) of the myocyte nuclei were diploid, 67.81 ± 6.62 were tetraploid and 10.3 ± 2.88 were octaploid. The comparative study carried out on 200 myocyte nuclei in tissue sections and in isolated cells of two normal hearts rendered similar results, and no significant difference was found between the data collected with both approaches.

4. Discussion

Our study shows that in human AMI, at the second week after diagnosis, more than 10% of the cardiomyocytes located at the border of the infarct are undergoing the cell cycle, as evidenced by the presence in the nuclei of the Ki67 antigen [11,12]. Given that in the normal control hearts the proportion of Ki67 positive cardiomyocyte nuclei was lower than 0.5%, a result similar to that of other report [13], it can be assumed that AMI induces the entrance of a significant number of periinfarct cardiomyocytes into the cell cycle. Apparently, this phenomenon is transient, because in hearts collected at earlier and later times after AMI the proportion of Ki67+ myocytes at the infarct border was significantly lower. This may be one of the reasons for the inefficiency of heart regeneration.

The transience of the described phenomenon has been also observed in rat infarcts, where the appearance of cardiomyocytes in cell cycle or mitosis is a temporal event lasting only a few days [14]. However, in the rat model the cycling and dividing myocytes were detected at earlier times...
than in human infarcts, and the number of Ki67+ cells was at least 10 times lower. This difference could be ascribed to the well-known fact that in the rat most adult myocytes are post-mitotic cells unable to replicate their DNA [15,16], a condition not present in humans. In fact, myocyte hypertrophy secondary to overwork is usually associated with an increase in the ploidy status, a situation that requires previous DNA replication [17].

The presence of sarcomeric α-actin in cycling or endomitotic cells provides strong evidence of their myocytic origin [2]. However, the highest number of myocyte Ki67+ nuclei as well as maximal chromosome condensation were observed at a time when heavy macrophage infiltration occurs and myofibroblast proliferation starts. Therefore, concomitant with the detection of the Ki67 antigen, we used immunohistochemical markers of those cells. Although we observed numerous Ki67+ macrophages and myofibroblasts, as well as mitotic bodies within them, myocytes were easily recognized on the basis of their sarcomeric α-actin reaction, in addition to their characteristic shape and the length of their short axis diameter. The possibility that the cycling myocytes could be originated from putative resident myocyte progenitors, recently described for the human heart [18], or from stem cells homing in the heart [19], appears unlikely on account of the large size of the cycling myocytes observed in our specimens. However, it cannot be discarded that cycling and endomitotic cardiomyocytes result from fusion with other cell types, such as adult stem cells, endothelial cells and fibroblasts, that retain their replicative ability, as has been recently suggested for murine hearts [20].

Although expression of the Ki67 antigen and phospho histone-3 indicates that the cardiomyocytes are in the cell cycle, it does not allow to establish if they are detained in the G1 phase or have progressed towards more advanced stages. Thus, we investigated in the 7–13 day-old infarcts, those with the highest number of Ki67+ myocytes, the presence of proteins that are expressed at specific moments of the cell cycle [21,22]. We observed that many myocytes located at the infarct border expressed cyclin D1 (present in the G1-S boundary), cyclin A (increased in the S phase) and cyclin B1 (essential for progression towards the G2-M phase).

However, despite the presence of proteins involved in the completion of the cell cycle and progression towards mitosis was documented, we could detect only three conventional cardiomyocyte mitoses in all the studied hearts. Since this observation was not coincident with a recent report claiming that in human AMI myocytes proliferate with relatively high mitotic indexes [2], we wondered which could be the reason for this discrepancy. Although the mean age of patients was 10 years lower, and the hearts were fixed earlier after death, the mean number of Ki67+ myocytes for all the hearts in both series was almost identical (4.84 vs 4). We thus speculated that the discrepancy could be ascribed to the different techniques employed for identification of mitosis rather than to differences in age or sample fixation. To test for this possibility, we studied successive tissue sections stained with fluorescent labels under confocal microscopy, and with immunoenzymatic methods under light microscopy. With fluorescent techniques we observed myocytes with condensed chromosomes arranged in metaphase and anaphase plates, indicative of outgoing mitosis, but when we employed immunoenzymatic methods and light microscopy, although we also observed many myocytes with condensed Ki67+ chromosomes presenting a distribution corresponding to the prophase, metaphase and anaphase stages of mitosis, the chromosomes were always contained within the boundary of an intact nuclear envelope. Since breakdown of this structure marks the end or prophase and initiation of metaphase, the presence of chromosomes arranged in metaphase and anaphase plates contained within a preserved nuclear envelope indicates that these cells are in endomitosis rather than in true mitosis. On account that the Ki67 antigen may occasionally adopt a punctuate or granular pattern that may be confused with condensed chromosomes, we considered that myocytes were in endomitosis only when the chromosomes were split in sister chromatids or were arranged in metaphase or anaphase plates, despite this requirement could lead to an underestimation of the real number of myocyte endomitosis.

Intranuclear mitosis and chromosome displacements with formation of chromosomes plates within an intact nuclear membrane have been described in some lower organisms [23, and in human trophoblast [24] and cancer cells [25].

As opposed to mitosis, endomitosis is an alternative mode of replication and distribution of the genetic material, and is one of the mechanisms responsible for polyploidy [26,27]. The most characteristic example of endomitosis in humans is the bone marrow megakaryocyte, a cell that leaves the diploid (2C) state to differentiate, synthesizing 4 to 64 times the normal DNA content within a single nucleus [28,29].

Our results, providing evidence of cardiomyocyte endomitosis, pose the possibility that this may be a frequent mechanism for replication of their genetic material, and that polyploidy is the consequence of this process, as has been hypothesized almost 30 years ago [30].

This is supported by the fact that in the samples collected after 2 weeks of evolution, when the number of myocytes in the cell cycle was very low, the nuclear DNA content of the myocytes adjacent to the infarct area was significantly higher than in those located in distant areas.

The facts that in the normal human left ventricle most of the myocyte nuclei are tetraploid [31,32], and that polyploidy is the rule in cardiomyocyte hypertrophy [33–36] and in cells surrounding myocardial scars [7,8], support the possibility that endomitosis could be a frequent phenomenon in the human heart.

The biological significance of polyploidy in hypertrophic cardiomyocytes is largely unknown. In addition to provide increased genetic material able to cope with the increased...
metabolic demands of a larger cell, it is known that ploidy regulates gene expression, and thus hyperploidy may control cell physiology, morphology and behavior by permitting the expression of genes that otherwise should not be expressed in diploid cells [37].

On the basis that the genetic program for reinitiating DNA synthesis exists in post-mitotic cardiomyocytes [1], that components of the cell-cycle machinery are involved in hypertrophic growth [38], and that, despite the discordance about its frequency, adult cardiomyocytes may enter in mitosis, if endomitosis is the usual replication mechanism of human adult cardiomyocytes, we think that efforts to induce karyokinesis and division into daughter cells should be addressed to determine why these cells, that apparently may arrive to the M phase, do not proceed towards a conventional mitosis, with formation of a mitotic spindle and breakdown of the nuclear envelope, but proceed instead towards endomitosis, with polyplody as the final result.

Although this may explain the absence of an efficient reparative process in the human heart, it also indicates that there is a potential to manipulate cell division, repair and proliferation. This is supported by recent reports from our laboratory, showing that VEGF gene transfer in a pig model of chronic ischemia induces an increase in the myocyte mitotic index [39] and myocyte hyperplasia [40].

References


