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The Meaning of Different Forms of Structural Myocardial Injury, Immune Response and Timing of Infarct Necrosis and Cardiac Repair

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Abstract: Although a decline in the all-cause and cardiac mortality rates following myocardial infarction (MI) during the past 3 decades has been reported, MI is a major cause of death and disability worldwide. From a pathological point of view MI consists in a particular myocardial cell death due to prolonged ischemia. After the onset of myocardial ischemia, cell death is not immediate, but takes a finite period of time to develop. Once complete myocytes' necrosis has occurred, a process leading to a healed infarction takes place. In fact, MI is a dynamic process that begins with the transition from reversible to irreversible ischemic injury and culminates in the replacement of dead myocardium by a fibrous scar. The pathobiological mechanisms underlying this process are very complex, involving an inflammatory response by several pathways, and pose a major challenge to ability to improve our knowledge. An improved understanding of the pathobiology of cardiac repair after MI and further studies of its underlying mechanisms provide avenues for the development of future strategies directed toward the identification of novel therapies. The chronologic dating of MI is of great importance both to clinical and forensic investigation, that is, the ability to create a theoretical timeline upon which either clinicians or forensic pathologists may increase their ability to estimate the time of MI. Aging of MI has very important practical implications in clinical practice since, based on the chronological dating of MI, attractive alternatives to solve therapeutic strategies in the various phases of MI are developing.

Keywords: Biomolecular mechanisms, cardiac repair, cellular mechanisms, histomorphological dating, myocardial infarction, therapeutic strategies.

INTRODUCTION

Although a decline in the all-cause and cardiac mortality rates following MI during the past 3 decades has been reported [1-4], MI is a major cause of death and disability worldwide. From a clinical point of view the term MI can be used when there is evidence of myocardial necrosis in a clinical setting consistent with acute myocardial ischemia [5, 6]. MI can be recognized by clinical features, including electrocardiographic findings, elevated values of biochemical markers of myocardial necrosis, and by imaging [5]. From a pathological point of view MI consists in a particular myocardial cell death due to prolonged ischemia. After the onset of myocardial ischemia, cell death is not immediate, but takes a finite period of time to develop. Once complete myocytes' necrosis has occurred, a process leading to a healed infarction takes place. In fact, MI is a dynamic process that begins with the transition froms reversible to irreversible ischemic injury and culminates in the replacement of dead myocardium by a fibrous scar [7].

The pathobiological mechanisms underlying this process are very complex, involving an inflammatory response by several pathways, and pose a major challenge to ability to improve our knowledge. As well as the definition of MI has important and immediate therapeutic implications, in the clinical practice the full comprehension of the repairing cardiac process following MI is of paramount importance for the development of potentially myocardial engineering-based therapies [8]. An improved understanding of the pathobiology of cardiac repair after MI and further studies of its underlying mechanisms provide avenues for the development of future strategies directed toward the identification of novel therapies.

This review retraces the pathomorphological mechanisms involved in evolving MI and their contributions to cardiac repair.

DIFFERENT FORMS OF STRUCTURAL MYOCARDIAL INJURY

The myocardial cycle of contraction – relaxation can be interrupted acutely in irreversible contraction or relaxation or chronically by a progressive loss of function, showing pathognomonic structural aspects. Apart from atonic death which is typical of MI and which will be discussed below, other morphological forms of myocardial necrosis exist, each of them bearing a different functional meaning. The different forms of myocardial injury have totally different structural, dysfunctional, and biochemical characteristics.

The myocardial cells can arrest in irreversible hypercontraction (tetanic death). The first histological change, visible

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within 10 min of onset, is an intense hypereosinophilia of the hypercontracted myocardial cells with rhexis of the myofibrillar apparatus into cross-fiber, anomalous, and irregular or pathological bands. Marked shortness of sarcomeres with a length much less than that observed in normal contraction and with a characteristic anomalous, extreme thickening of Z lines are the morphological hallmarks of this model of myocardial death. This myofibrillar rhexis is probably due to the mechanical, rhythmic action of the normal contracting myocardium which surrounds rigid hypercontracted elements and may range from a few contraction bands to total granular destruction of myofibrils (myofibrillar degeneration). Repair of the pancellular lesion is by macrophagic digestion of all structures within the sarcolemmal tubes (alveolar pattern) followed by a progressive collagenization. The other pattern is characterized by a unique band of 10 – 20 hypercontracted sarcomeres close to the intercalated disc (paradiscal lesion). This band does not show rhexis of myofibrils and may assume a dark, dense, ultrastructural aspect or a pale, clear one, with very thin Z-lines and myofibrils, and mitochondria "squeezed" in the normal portion of the myocyte. The paradiscal lesion does not show any macrophagic infiltrates [9, 10]. This model of death (coagulative myocytolysis or contraction band necrosis, CBN) is experimentally reproduced by intravenous catecholamine infusion and we consider it an important histological hallmark of adrenergic stress linked with peroxidation caused by a variety of mechanisms, intrinsic or extrinsic to the heart [10-13]. In the literature CBN has been considered an ischemic change since it is found associated with and is reproduced by experimental reperfusion. This impression may have been induced by animal models of permanent and temporary coronary occlusion. From experience with the dogs, a coronary occlusion of the left circumflex branch of 60 min duration produces a small subendocardial infarct characterized by stretched myocells with prominent I-bands. However when the coronary occlusion lasts only 40 min followed by 20 min reflow, the histological pattern transforms into typical CBN that was interpreted as ischemic. In further experiments by prolongation of occlusion and/or reperfusion time, transmural (wavefront phenomenon) myocardial changes mainly formed by CBN associated with marked interstitial hemorrhage were obtained [10, 14-16]. The lesion is unrelated to ischemia. Its presence in acute coronary syndromes is probably due to catecholamines released within the myocardium as a reflex response [17] to regional asynergy of the infarcted or preinfarcted zone, a hypothesis that is supported by the abolishment of contraction bands and ventricular fibrillation with beta-blocking agents in experimental MI and in reperfusion necrosis. They may trigger a catecholamine myotoxicity linked with ventricular fibrillation and acting through free radical mediated lipid peroxidation with intramyocellular Ca^{2+} influx. Contrary to the general opinion that excess catecholamines produce cardiotoxicity mainly through binding to adren-oceptors, there is increasing evidence that catecholamine induced deleterious actions may also occur through oxidative mechanisms [18, 19] which undoubtedly occur during myocardial reperfusion after ischemia [20-24].

The failing death of myocells (colliquative myocytolysis) is characterized by progressive loss of myofibrils paralleled by intramyocellular edema. This process starts around apparently normal nuclei with myofibrillar disappearance producing an increasing vacuolization of myocardial cells until a histologic pattern of empty sarcolemmal tubes without any cellular reaction or signs of healing results [25]. Myocytolysis or vacuolization is often interpreted as a histological sign of myocardial ischemia; colliquative myocytolysis is the histological hallmark of congestive heart failure, independent of its underlying cause; including acute MI in which colliquative myocytolysis expresses a secondary nonischemic complication involving subendocardial and perivascular myocardium preserved in infarct necrosis [26, 27].

PATHO-MORPHOLOGY OF ACUTE MYOCARDIAL ISCHEMIA

Myocardial infarct necrosis is caused by a reduction below a critical point of the nutrient blood flow. More than 95% of the energy required for cardiac myocyte function is derived from oxidative phosphorylation. Interruption of blood flow to the myocardium disrupts oxygen supply, triggering rapid declines in ATP and increased AMP/ATP ratios. Brief episodes of transient myocardial ischemia are tolerated by myocytes. Experimental studies performed in canine heart, show that coronary occlusions of up to 15 minutes result in reversible injury, and beyond that, irreversible injury [28, 29]. In humans, irreversible ischemic damage of the myocardium begins after 20 minutes of total ischemia [30], starting from subendocardium and progressing into the subepicardium of the ischemic myocardial bed-at-risk, such that the wavefront of irreversible injury is completed after 3 to 4 h or less [15, 31-34].

The metabolic changes associated with the sudden onset of ischemia caused by occlusion of a major coronary artery include (a) cessation of aerobic metabolism, (b) depletion of creatine phosphate (CP), (c) onset of anaerobic glycolysis, and (d) accumulation of glycolytic products, such as lactate and alpha glycerol phosphate (alpha GP), and catabolites of the nucleotide pools in the tissue [7, 14, 35, 36]. Restoration of the blood flow can, paradoxically, trigger several physiopathological events that can exacerbate tissue injury and reduce the beneficial effects of reperfusion, leading to cell death of critically injured cardiomyocytes (lethal reperfusion injury) [37-47].

The evolving process of myocardial ischemic injury is a highly orchestrated process in which several important morphofunctional events occur that consequently lead to the removal of the injured tissue and the establishment of a scar [48-58].

Cell Death

The loss of the cardiomyocites constitutes the first event and it represents a signal for a cascade of pathophysiological events; in experimental models of MI a large burst of cell death takes place within the ischemic area over the first 6 to 24 hours [59]. Cardiomyocytes' death occurs *via* necrosis and *via* apoptosis [60, 61]. Although MI was long considered to be characterized by nonapoptotic ("necrotic") cell death due to the breakdown of cellular energy metabolism, since Gottlieb documented reperfusion-induced apoptosis in rabbit cardiomyocytes [62] there has been growing evidence that hypoxia activates the suicide program of cardiac myocytes

in vitro [63] and *in vivo* [64] and that myocyte loss during the acute stage of myocardial MI involves both apoptotic and nonapoptotic cell death [65-73]. However, the conclusions drawn by all the studies on this matter seem quite contradictory.

Experimental studies performed on rats showed a significantly greater number of cardiomyocytes undergoing apoptosis than necrosis and that apoptotic myocyte cell death preceded cell necrosis and is the major determinant of infarct size [59, 74, 75]. These Authors concluded that programmed myocyte cell death is the prevailing form of myocardial damage, whereas necrotic myocyte cell death follows apoptosis and contributes minimally to the progressive loss of myocytes after infarction. Apoptosis was reported to be the major form of cardiomyocyte death up to 6 h after coronary occlusion in rats [59]. Conversely, other Authors [62, 76, 77] hypothesized that apoptotic cell death is initiated by ischemia but that reperfusion is needed for completion of the apoptotic cascade. Studies performed on adult rat cardiomyocyte culture [65, 78] suggested that apoptosis is a predominant mode of cell death during reoxygenation, but nonapoptotic cell death predominates during prolonged hypoxia alone. Reoxygenation, although associated with both apoptotic and nonapoptotic cell deaths, induced significantly greater apoptosis than hypoxia alone, despite the fact that hypoxia alone induced more overall cell death. Other studies [79, 80] reported apoptosis to contribute 5% to 33% of cardiomyocyte loss in various animal models of myocardial ischemia and reperfusion.

In humans, DNA fragmentation was detected in cardiomyocytes from hearts autopsied following fatal MI [66] and subsequently many Authors investigated the models of cardiac myocytes' death in human infarction [68, 81]. However, the differential contribution of necrosis and apoptosis in myocardial ischemia/reperfusion injury is still unclear and there is controversy whether the biologic form of cell death is "apoptotic" or "nonapoptotic" [43, 45, 82-104]. It has been strongly underlined that the simple use of TUNEL-positivity and DNA ladder detection for determination of apoptosis can result in misunderstandings as to the mode of cell death [100, 105-111]. Takemura *et al.* using electron microscopy to assess apoptotic morphology, particularly preservation of membrane integrity, found no cardiomyocytes exhibiting apoptotic ultrastructure in infarcted areas, thus concluding that although some final steps in the apoptotic process may be activated in infarcted tissue, this activation likely has no relevance to the extent of infarction already determined by irreversibly oncotic cardiomyocytes [112]. Other Authors reported similar results and light and electron microscopic evidence of typical apoptotic morphology in cardiomyocytes in *in vivo* models of myocardial ischemia has been scant [113, 114]. Studies by Nakagawa *et al.* [115] supported the doubt of cardiomyocyte apoptosis during ischemia/reperfusion. Recently Konstantinidis *et al.* [83] investigated the mechanisms of cell death in MI and underlined that apoptosis and necrosis are mediated by distinct, but highly overlapping central pathways; the extrinsic pathway (death receptors DRs) and the intrinsic (mithocondrial/endoplasmic reticulum ER) one, in fact, appear to be linked by multiple biochemical and functional connections. Some death ligands may induce apoptosis or necrosis depending on the downstream events; mithocondria and ER activation are central to both apoptotic and necrotic process. The Authors concluded that both apoptosis and necrosis are involved in MI [83]. Other Authors had previously postulated such an hybrid ischemic injury model in which both apoptotic and oncotic mechanistic pathways can be activated in the same cardiomyocytes [43, 99].

The issue of the mode of death of ischemic cardiomyocytes is even more complex if one considers that dead cells are so severely degraded by the final stage that it cannot be morphologically determined whether they died *via* apoptosis or necrosis, and that necrosis refers only to an irreversible stage of cell death, even though dying cells generally progress from a reversible to an irreversible stage [112]. Due to these observations, Majno and Joris [116] revived the term "oncosis" to identify cell death accompanied by swelling and substituted oncosis for necrosis in cells dying *via* a process involving cellular swelling. In conclusion, contrasted to apoptosis which is a programmed form of cell death, many Authors prefer the term "oncosis" to identify a model of cell death as passive response to external noxae, including ischemia while necrosis is the final irreversible phase of cellular death in which advanced cellular degeneration is seen regardless of the mode of death [112]. In their review Buja *et al.* [99] identified the oncotic process as evolving from a reversible phase, involving mild alterations in ionic transport systems, to an irreversible stage with physical disruption of the cell membrane. These stages of oncotic membrane injury are accompanied by progressive morphologic changes of organellar and cell swelling, membrane blebbing, and membrane and cell rupture with leakage of intracellular constituents that provokes the response of exudative inflammation [117]. On the other hand, key morphological features of apoptotic death are represented by shrinkage of the nucleus with condensation and fragmentation of the chromatin (pyknosis) followed by fragmentation of the nucleus (karyorhexis) and cytoplasm into apoptotic bodies which are rapidly phagocytosed by macrophages or occasionally by adjacent cells. When this process is efficient, inflammation is avoided [118] (Fig. **1**).

Finally, it is noteworthy that the mechanisms involved in cellular death are multifaceted and complex, since reperfusion injury can be responsible for a significant proportion (one-third or more) of cell death (either necrosis or apoptosis) [38]. Reperfusion induces abrupt biochemical and metabolic derangements in cardiomyocytes already perturbed by the effects of acute ischemia. Mitochondrial reenergization, the generation of reactive oxygen species (oxygen paradox), intracellular calcium overload (calcium paradox), and the rapid restoration of physiological pH (pH paradox), collapse of ATP production, loss of mythocondrial integrity subsequent to opening of the mitochondrial membrane PTP, and sarcolemmal disruption are thought to be deleterious effect of reperfusion [20, 21, 44, 119].

Histological and Immunohistochemical Findings in Early Infarction

From a morphological point of view, different findings have been described in the early phase of MI. The earliest histological signs are visible within 30 min of infarct onset

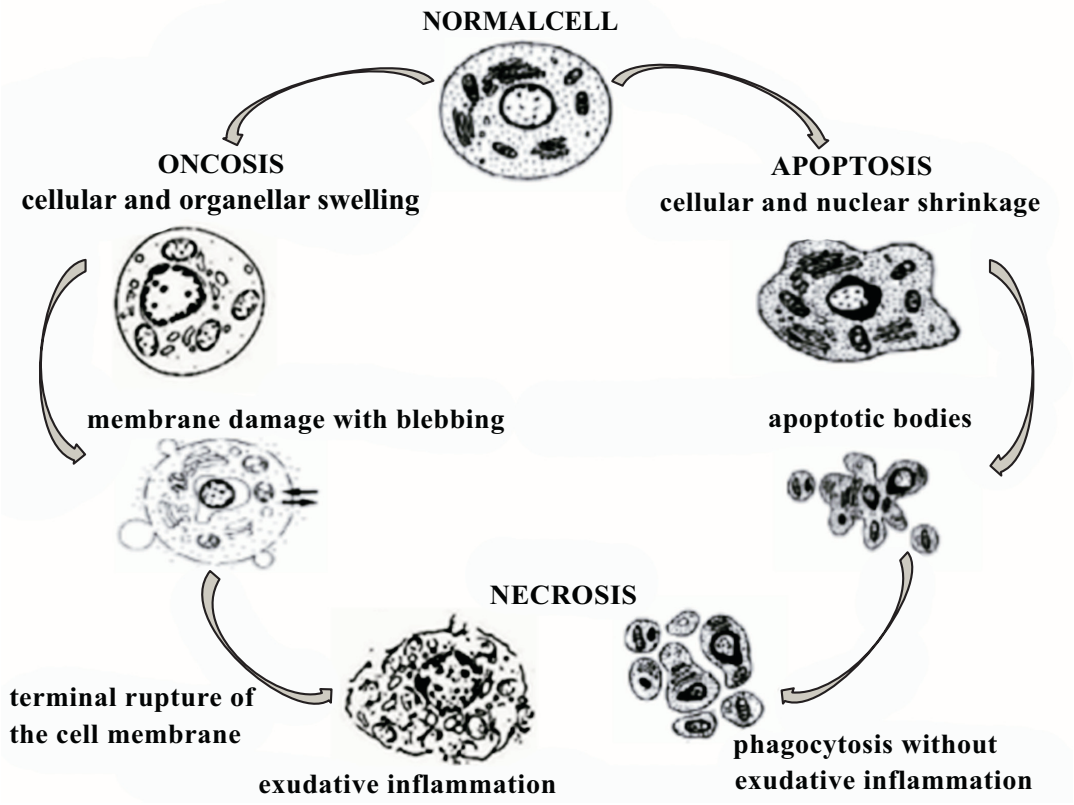

Fig. (1). models of cellular death (modified from Buja LM, Eigenbrodt ML, Eigenbrodt EH. Apoptosis and necrosis. Basic types and mechanisms of cell death. Arch Pathol Lab Med 1993; 117(12): 1208-14).

and consist of mild myofiber eosinophilia and elongation of sarcomeres and nuclei. Functionally the loss of contraction of a myocardial region is the first change following MI (atonic death). Swelling of the entire cytoplasm and changes of the mitochondria with swelling and dissolution of the cristae mitochondriales have been detected by electron microscopy up to 30 minutes from MI [120] with subsequent cellular membrane blebbing and complete cell rupture. In the myocardial interstitium, after 20–24 minutes from MI, increased vascular permeability adds to the increased intercellular oncotic pressure; interstitial edema becomes evident after 8 h [121].

One of the earliest (within minutes) histological sign observed in the infarcted area is prominent CBN [122-124]. When myocardial ischemia is brief enough to cause the death of only a part of the myocytes within the myocardium at risk (severely ischemic), cell death occurs almost exclusively during the first minutes of reperfusion in the form of CBN. Strikingly, hypercontracted, dead cardiomyocytes are not scattered across reperfused myocardium, but are invariably connected to other dead myocytes within well-delimited areas of contraction band necrosis, often with irregular geometry [125]. This pattern cannot be explained as a consequence of microvascular or collateral distribution or other structural patterns, and computer simulation studies indicated that it is due to some kind of cell-to-cell interaction [126, 127]. Traditionally interpreted as an ischemic myocardial lesion, this phenomenon has been ascribed to a rapid re-energisation of myocytes with calcium overload and may be related to adrenergic stress [122, 128]. Reperfused myocardium is often reddish and hemorrhagic due to microvascolature damage which is documented to occur later than cardiomyocytes injury (45-60 min) [123, 129-133].

The usefulness of immunohistochemical markers for the diagnosis of early ischemic myocardial damage has been suggested many years ago because most of them can be detectable as early as few minutes after the beginning of the myocardial injury, even before myocardial ischemia is visible macroscopically or histologically. Immunohistochemistry is an appropriate procedure to evaluate cell recruitment and humoral network in myocardial response to ischemic insult. Cellular and plasma markers have, traditionally, been selected on the basis of their different diagnostic potential in early ischemic myocardial injury (C5b-9 complex, C9, fibronectin and fibrinogen, myoglobin, cardiac troponin C and cardiac troponin T, desmin) [134-141]. Plasma markers (C5b-9 complex, fibronectin) tend to accumulate in necrotic cardiac cells and interstitium and stain positive in ischemic areas while cellular markers (such as myoglobin and cardiac troponin) show an early depletion from ischemic areas and, usually, appear in very high serum concentration [139]. Generally, the loss of cellular antigen (negative markers of necrosis) is detectable earlier than the accumulation of the cellular antigens (positive markers of necrosis) [136]. C5b-9 complement complex was considered a specific marker for necrosis which allowed detection of a single – cell damage and whose specificity was not reduced due to putrefaction [141]. The detection of the complement complex C5b-9 becomes positive within 30 – 40 minutes from myocardial ischemia [141]; however the study by Ortmann *et al.* [136]

showed that fibrinogen and fibronectin start to become positive later that the cellular antigens but earlier than C5b-9 [136,139]. Products of complement activation in MI (*e.g.* C4d, C9) have been investigated in fatal human cases of MI [142] resulting an immunoreactive response for C4d and C9, with clear delineation between necrotic and viable myocytes, in all the infarctions with evidence of cellular injury but without a polymorphonuclear infiltrate.

Ongoing Phases of Infarct Healing

MI triggers a reparative response in which overlapping phases are detectable (Fig. **2**).

Following to cell disintegration an intense inflammatory response by activating innate immune mechanisms is elicited [48, 143]. A great mass of studies have demonstrated the role of humoral (cytokines and inducible chemokines, complement, and toll – like receptors) and cellular (monocytes, macrophages, dendritic cells, T cells, mast cells, platelets, endothelial cells) mediators in the initial healing phases following cardiomyocytes' death [48, 143-152].

Neutrophils accumulate in the infarcted myocardium in the first hours after onset of ischemia, and peak after one day; thereafter, monocytes and their lineage descendant macrophages dominate the cellular infiltrate [153]. In this phase an up – regulation of several cytokines (*e.g.*, Interleukin 1β, Interleukin 18, Interleukin 6, Tumor Necrosis Factor α, *etc.*), chemokines (*e.g.* Interleukin 8, MCP-1/CCL2), and adhesion molecules (*e.g.* ICAM 1, E selectin) occurs [152, 154, 155]. The inflammatory cells release proteolytic enzymes and reactive oxygen species (ROS) that harm myocytes that survived the ischemic period. The first peripheral leucocyte reaction (4-7 h) gradually evolves to a strong evidence (9 h) with further leucocyte penetration of the infarct area (18-24 h). The penetration of leucocytes continues for 5-6 days and then inflammatory cells disappear within weeks from infarct [120] as expression of pro-inflammatory mediators' suppression [143]. Other authors hypothesized that disappearance of inflammatory cells is due to their programmed death [50]. Cardiac mast cells rapidly degranulate after MI and release a wide variety of mediators with pleiotropic actions: histamine that induces surface expression of P-selectin in endothelial cells and facilitates the recruitment of rolling leukocytes; tryptase that incites granulocyte recruitment and upregulates cytokine and chemokine synthesis, TNF-α that interferes in the cytokine cascade [48].

At the periphery of the necrotic myocardium, a repair process starts by neutrophilic and macrophagic digestion of tissue. The trigger for the proliferative phase is represented by the release of anti – inflammatory citokynes (such as Interleukin 10 and growth factors, such as Transforming Growth Factor β (TGF – β) (so called stop signals) leading to the formation of highly vascularized granulation tissue; at this phase of the healing process expression of pro – inflammatory mediators ceases and fibroblast – like cells and endothelial cells proliferate. A vascular network begins to form at the infarct site on day 3 postMI that nourishes myofibroblasts (MyoFb) and provides for their metabolic activity [50, 154, 156, 157].

At the same time fibroblasts are stimulated to differentiate in MyoFb [158]; in addition to resident fibroblasts other sources of MyoFb are invoked: i) epithelial and endothelial cells can adopt a myofibroblast phenotype through a transi-

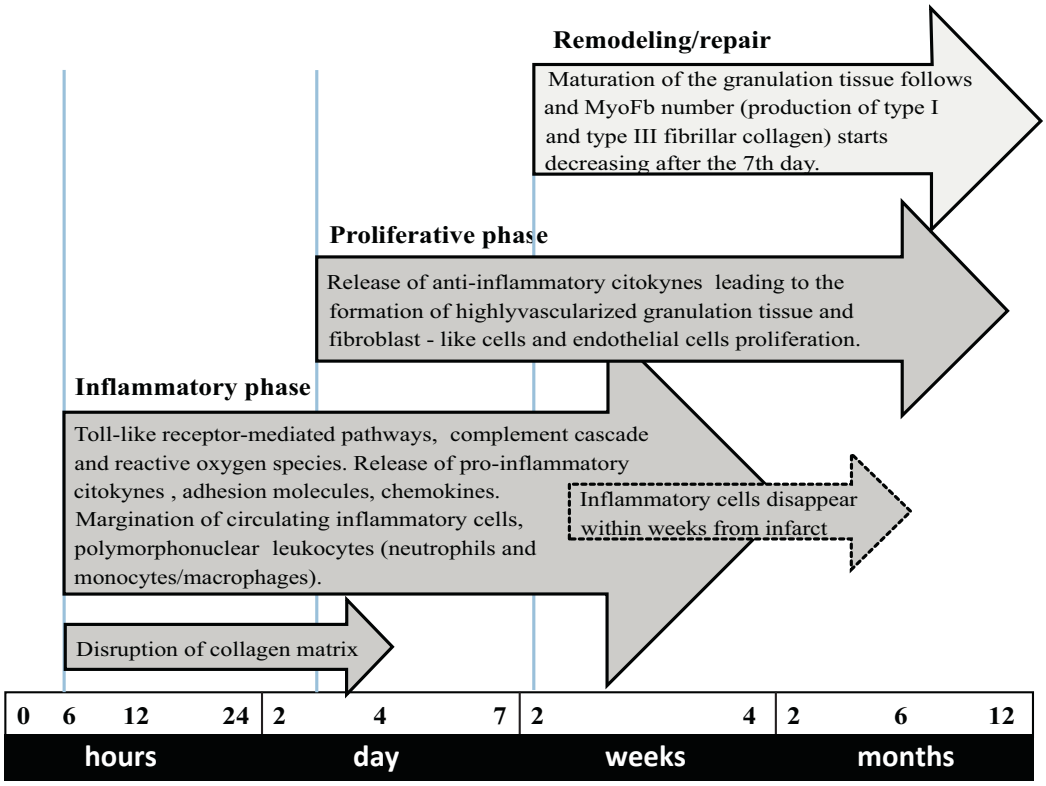

Fig. (2). ongoing phases of myocardial infarct.

tion process (endothelial – mesenchymal transition and epithelial – mesenchymal transition); ii) fibroblast – like cells are thought to be derived from bone – marrow stem cells (fibrocytes); iii) MyoFb can originate from pericytes, extensively branched cells located in capillaries and small blood vessels that can dissociate from the walls of the vessels, migrate and differentiate into the myofibroblast phenotype [60, 159, 160]. These cells play a major role in scar formation; they are found at the infarct site soon after the arrival of inflammatory cells and they are responsible for the production and deposition of collagen and other proteins of the extracellular space [60, 161-165]. A strict cross – talk between cardiac myocites and myofibroblasts is critical in the response to ischemic injury [166, 167].

As some Authors have underlined besides the pivotal mechanical role of cardiac extracellular matrix (ECM), matrix components have a dynamic role in regulation of inflammatory and fibrotic signals in the infarcted area [168]. During the inflammatory phase of infarct healing an early disruption of collagen matrix is present [169, 170] due to the enhancement of Matrix Metalloproteinases (MMPs) expression by proinflammatory mediators such as TNF – α and IL - 1β [171, 172]. MMPs are an endogenous family of enzymes that have been identified to be responsible for collagen matrix remodeling in a number of physiological processes. Experimental studies performed on pigs demonstrated that an early onset of MMP activation occurred within the interstitium of the MI region and that, with longer periods post MI, this occurred also in the remote regions of MI [173]. Generation of matrix fragments activates a cascade of events such as neutrophil, monocyte and fibroblast chemotaxis. The matrix alterations during the proliferative phase of healing provide essential signals for MyoFb activation, matrix organization, and repression of the inflammatory reaction [168]. In the maturation phase of infarct healing, the strict cross – talk between matrix and MyoFb persists: "stress – shielding" of the myofibroblasts by the cross – linked matrix and growth factor withdrawal may induce quiescence and ultimately cause apoptotic death [164, 168, 174].

Histological and Immunohistochemical Findings in Healing Infarct

Inflammatory Phase

Typical early changes detectable in the inflammatory phase occur approximately 6 to 8 hours after an infarct in human hearts with a margination of circulating inflammatory cells, polymorphonuclear (PMN) leukocytes that include neutrophils and monocytes/macrophages, in vessels at the periphery of the necrotic zone followed by an infiltration of these elements, without fibrin or haemorrhage, into the ischemic issue. A crowd of PMN is visible along a line between infiltrated and noninfiltrated necrotic myocardium in large areas of necrosis.

Before the influx of the inflammatory cells becomes histologically detectable, the presence and the nature of the immuno-inflammatory and cellular phenomena accompanying the cardiac alterations during inflammatory phase of MI can be evaluated by immunohistochemistry. Immunohistochemical analyses on experimental MI in mice have been performed [175], aimed to distinguish the different clusters of cellular population T and the appearance of the humoral factors in the infarcted regions. To the best of our knowledge studies focusing on the application of immunohistochemistry in assessing the timing of human infarcts are unavailable in the literature. The current knowledge about the chronology of the responses of myocardial tissue following the occurrence of an ischemic/reperfusion insult, as well as our previous experience both in *in vivo* animal models [176] and in human diseases [177] using immunohistochemistry and immunoblot analysis to detect the expression of inflammatory cytokines, induced us to apply these techniques on cardiac tissue specimens of fatal MI. We investigated samples of cardiac tissue obtained during post-mortem examinations of subjects died from MI, using a panel of antibody (CD15, IL-1β, IL-6, TNF-α, IL-15, IL-8, MPC-1, ICAM-1, CD18, anti - tryptase) (Table **1**). Our preliminary unpublished results (semi-quantitative analysis) demonstrated a mild positivity of CD15, tryptase, IL-1 β, IL-6, TNF-α, IL-8, MPC-1, and tryptase reaction in the infarcted

Table 1. Semi-quantitative evaluation of the timing related immunohistochemical findings.

Antibody	Very early infarction (0-6 hours)	Early infarction (6-12 hours)
Tryptase	+/++	+++
TNF-α	+/++	+++
CD15	+/++	+++
IL-1β	++	+++
IL-6	++	+++
IL-8	++	+++
IL-15	+++	+++
MPC-1	++	+
ICAM	+	++

(-): not expressed; (+): isolated and disseminated expression; (++): expression in groups or widespread foci; (+++): widespread expression.

zone matched by the immunodepletion of negative markers of necrosis (such cellular antigen troponin) and in the absence of histological signs of cellular margination (approximately 4-6 hours from ischemia). In older infarction (8-12 h) a progressively stronger immunoreactions for the same antibodies was visible in areas where the margination of circulating inflammatory cells became histologically detectable up to a very strong expression in the oldest ones (> 12 hours) (Figs. **3**, **4** and **5**).

Although further studies are needed, these preliminary results led us to consider the immunohistochemical study of human infarctions' tissue as a matter of paramount utility in detecting very early infarction, thus integrating the traditional microscopic examination of the heart and allowing research on MI timing to advance significantly.

Proliferative Phase

Starting from 2-3- weeks from MI pronounced peripheral granulation tissue with sprouted capillary blood vessels, fibrocytes, fibroblasts, lymphocytes, few plasma cells, macrophages, possibly siderophages, and few granulocytes become increasingly apparent. The granulation tissue phase can extend for approximately 1-2 months in humans [61]. From 5

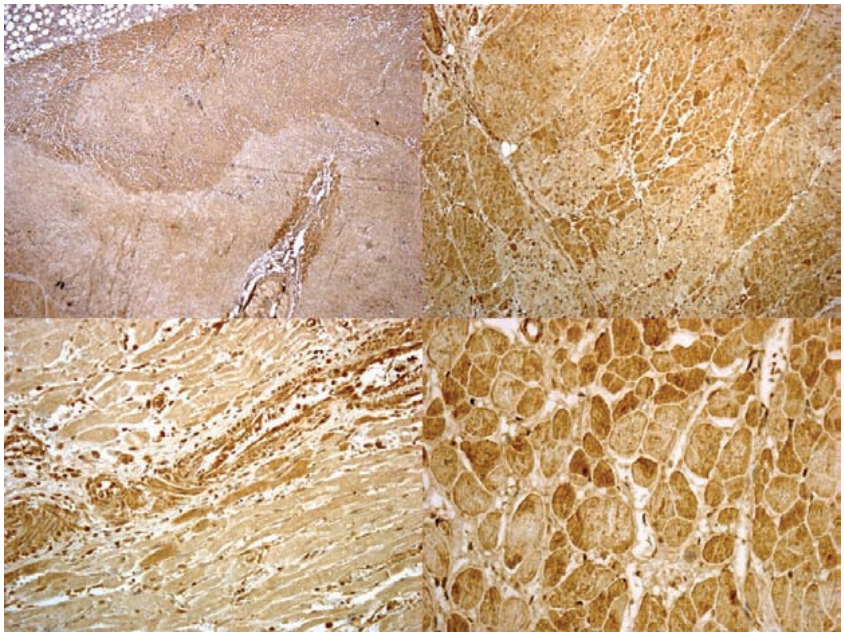

Fig. (3). Immunohistochemical detection of the time course of the IL-15: IL-15 at 1 hr (**A**-**B**), after 3 hrs (**C**), and 6 hrs (**D**). Reactions may be interpreted as the adaptive response of jeopardized myocardium with respect to the cardiac dysfunction resulting from myocardial infarction.

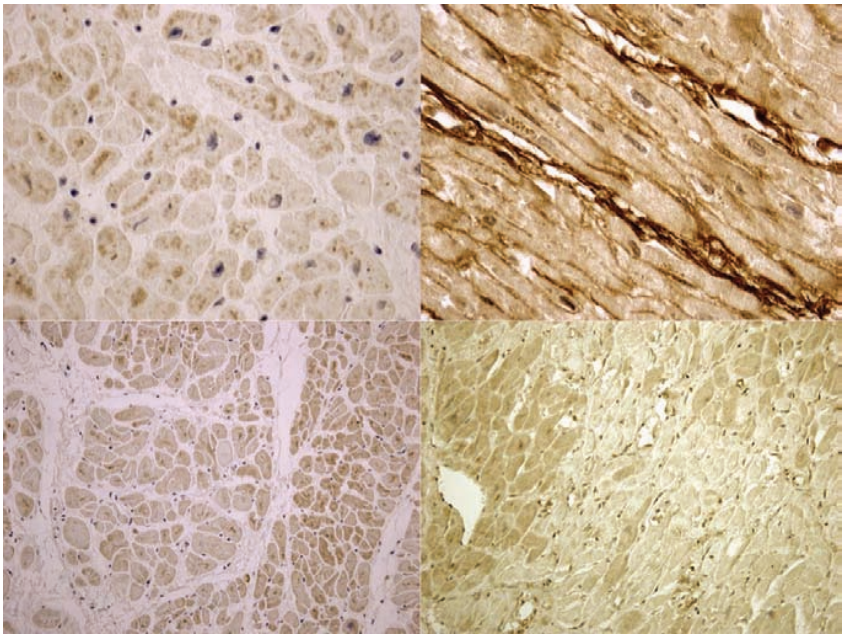

Fig. (4). Immunohistochemical detection of the time course of the cardioinhibitory cytokines: (**A**) IL-1β, (**B**) IL-6, (**C**) IL-8 during the very early phase of MI. MCP-1 expression: 4 hrs after MI (**D**).

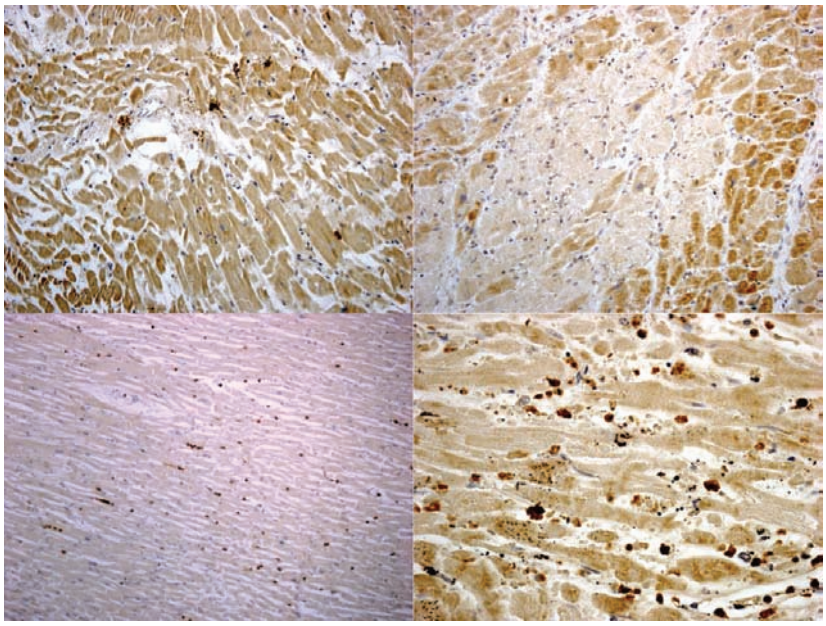

Fig. (5). (**A**) mast-cells reaction after 8 hrs (red circles). TNF-α expression after 6-8 hrs (**B**). CD15 after 6 hrs (**C**) and 12 hrs (**D**).

weeks to 2-3 months collagen fiber or scar tissue with endothelially coated blood vessels of varying density, siderophages still possible, loose infiltration with lymphocytes, few plasma cells, scant granulocytes are the histological findings observed [120]. Maturation of the granulation tissue follows and MyoFb number starts decreasing after the 7 th day post reperfusion even if it is demonstrated that they may persist in the healed area up to 20 years after MI [178]. This suggest that MyoFb play an important role in maintaining the stability of the scarred area by continuing the production of type I and type III fibrillar collagen long after scar tissue have replaced the necrotic tissue. Well – healed infarcts contain large amounts of ECM, which can occupy up to 90% of the healed area [61].

Up to 3-6 months scar tissue with fewer cells, few capillary blood vessels, scant siderophages are the predominant histological findings [120].

Recently, Tatìc *et al.* [179], investigated the histological, histochemical and immunohistochemical findings in cardiac samples taken from 177 patients who had died of acute myocardial MI. Interestingly, in the scar, a large number of cells of various size and form (spindle, oval, elongated with abundant cytoplasm, small with one nucleus and cells with scanty cytoplasm) were found. Histochemical and immunohistochemical analyses revealed that large oval cells showed negative reaction to lymphocytic and leukocytic markers, and positive to alpha actin, actin HHF35, Ki-67, myosin, myoglobin and desmin. Elongated cells were also positive to those markers. Small mononuclear cells showed positive reaction to lymphocytic markers. Endothelial and smooth muscle cells in the blood vessel walls were positive to CD34 and CD31, and smooth muscle cells to actin. Oval and elongated cells were positive to Proliferating cell nuclear antigen (PCNA) and Ki-67. The preserved muscle fibers in the scar were positive to myosin, myoglobin and desmin as well as elongated and oval cells. The Authors' conclusions that the myocardium is not a static organ without capacity of cell regeneration are in line with the affirmation that infarct scar is now recognized as living tissue: composed of a persistent population of fibroblast-like cells whose ongoing activity includes a regulation of collagen turnover and scar tissue contraction and which are nourished by a neovasculature [50].

Ventricular Remodeling

Over the years, it has become increasingly appreciated that myocardial infarcts, particularly large transmural infarcts, may result in complex alterations in ventricular architecture involving both the infarcted and noninfarcted zones (ventricular remodeling) and that long-term outcome of infarcted patients largely depends on the extent of post-infarct remodeling [180, 181]. Adverse ventricular remodeling after MI is responsible for most of heart failure cases. Post - infarct remodeling is a dynamic process that involves a considerable number of biomolecular events, such as cell death and survival, oxidative and mechanical stress, hemodynamic change, inflammatory reaction, neuroendocrine activation, changes in the extracellular matrix, and fibrosis [56, 152, 182-185]. An optimal balance between the formation of an early mature scar and an excessive fibrotic response is of paramount importance for the preservation of ventricular geometry and function post MI [186]. A pathophysiological underpinning of the LV remodeling process is that continuous changes occur in the structure and function of the fully perfused myocardium surrounding the infarct region, described as the borderzone myocardium. Extension of these changes from the borderzone to contiguous normal myocardium is a process defined as infarct expansion towards the epicardium during the first few hours after reperfusion. The infarct border zone, which is located between the infarct and

remote zones, represents a cornerstone in limiting the infarct expansion [187, 188]. The mechanical shear stress imposed on cardiomyocytes lining the infarct scar induce oxidative stress and activate pro-inflammatory pathways within these cells. Expression of both TNF-alpha [189] and iNOS protein [188] have been documented in cardiomyocytes bordering the infarct scar [186]. Recently, for the first time a proteomic analysis specifically using myocardial tissue from the border zone during the early stage of post-infarct remodeling has been performed to test the hypothesis that functional proteins could be differentially expressed and might play significant roles in regulating the dynamic process of ventricular remodeling [190]. A differential myocardial proteome profile was identified in the border zone during early stage post-infarct remodeling.

CONCLUSION

The chronologic dating of MI is of great importance both to clinical and forensic investigation, that is, the ability to create a theoretical timeline upon which either clinicians or forensic pathologists may increase their ability to estimate the time of MI. Traditional dating of MI, based on histological findings such as cellular margination, is not so useful for clinical and forensic purposes because very early infarction cannot be distinguished with any degree of certainty. The application of selective immunohistochemical techniques can open up a new field of investigation in the issue of determining myocardial infarct age. Besides routine histological techniques, the immunohistochemical investigation of many bioactive substances essentially involved in the response to myocardial ischemia, may give a substantial contribution to myocardial infarct's age estimation (Table **2**).

Aging of MI has very important practical implications in clinical practice since, based on the chronological dating of MI, attractive alternative to solve therapeutic strategies in the various phases of MI are developing. The target of early management of acute MI is reperfusion therapy which can alter the course of infarction, limit the extent of myocardial damage, and improve subsequent prognosis. The efficacy of reperfusion therapies is decreased with the prolongation of the time interval between the onset of symptoms and treatment [191]. Knowledge on the pathophisyological mechanisms underlying to the evolving process of MI presents a unique therapeutic challenge to clinicians.

An ever-growing volume of studies over the past 30 years speaks to the recent and rapid growth in targeting the immune response following MI in order to optimize cardiac repair [48]. Cardiac stem cell therapy to modulate inflammation upon MI may represent a promising approach in cardiovascular medicine [192-195] and tissue engineering has emerged as an alternative cell-based approach, aiming at partial or full replacement of damaged organs with *in vitro* generated tissue equivalents [8, 196-199].

Table 2. Histological/immunohistochemical age determination of MI and cardiac repair (modified from Dettmeyer RB. Myocardial Infarction. In: Dettmeyer RB, Ed. Forensic Histopathology. Springer-Verlag: Berlin Heidelberg, 2011; pp. 245.

Cell death	**Up to 30 minutes – 1 hour**	Cytoplasm and mitochondrial swelling and dissolution of the cristae mitochondriales (electron microscopy); loss of contraction with stretching of the myocardium in flaccid paralysis, resulting in a very early elongation of sarcomeres and nuclei; mild myofiber eosinophilia. Contraction band necrosis. At immunohistochemistry loss of cellular antigen (myoglobin and cardiac troponin) is detectable earlier than the accumulation of plasma markers (C5b-9 complex, fibronectin).
Inflammatory phase	**4-6 hours**	Mild positivity of immunoreaction (tryptase, CD15, IL 1-β, IL – 6, IL -8, IL – 15, TNF –α, MPC – 1) in areas where depletion of cellular antigens (myoglobin and cardiac troponin) is detectable within 30 – 40 minutes from ischemia.
	6-8 hours	Necrosis of the infarcted area becomes more evident; a crowd of polymorphonuclear leucocyte infiltration from the periphery is evident. General and intense eosinophilia of myofibers. Interstitial oedema. Immunopositivity to the antibodies anti tryptase, CD15, IL 1-β, IL – 6, IL -8, IL – 15, TNF –α, MPC – 1 becomes stronger and ubiquitously widespread.
	8-12 hours	Pronounced necrosis of the infarcted areas; strong evidence of PMN margination with further leucocyte penetration of the infarct area. Strong immunopositivity to the above mentioned antibodies.
	18-24 hours	Pronounced necrosis, further leucocyte penetration of the infarcted area.
Proliferative and maturation phases	**5-7 days**	Inflammation cells disappear; fibroblast – like cells and endothelial cells proliferate. Initial formation of peripheral granulation tissue. Immunopositivity to the antibodies anti-IL10.
	2-3 weeks	More pronounced peripheral highly vascularized granulation tissue with sprouted capillary vessels, fibroblasts, lymphocytes, few plasma cells. Macrophages, possibly siderophages, few granulocytes.
	5 weeks- 2/3 months	Collagen scar with endothelially coated capillary blood vessels, siderophages still possible, loose infiltration with lymphocytes, few plasma cells, scant granulocytes.
	3-6 months	Scar tissue with fewer cells, few capillary blood vessels, scant siderophages.
	6-12 months	Fibroblasts and vascular cells progressively disappear and a prominent collagen-based scar is present.

In the very near future, proteomics may help clinicians and pathologist to better understand mechanisms related to cardiac repair and remodeling and provide targets for future therapies [200-203]. In addition, these technologies might be used as a tool for optimizing individual treatment programs [204].

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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