Myocyte Death, Growth, and Regeneration in Cardiac Hypertrophy and Failure

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Abstract—The accepted paradigm considers the adult mammalian heart as a postmitotic organ, which possesses a relatively constant number of myocytes from shortly after birth to adulthood and senescence. This notion is questioned by the demonstration that although most adult myocytes are terminally differentiated, there is a small and continuously renewed subpopulation of cycling myocytes produced by the differentiation of cardiac stem-like cells. Myocyte death and myocyte regeneration are introduced as major determinants of cardiac homeostasis and alterations of ventricular anatomy and function in physiological and pathological states. The possibility of reconstituting dead myocardium by stem-like cells is advanced and proposed as a major area of future research. (Circ Res. 2003;92:139-150.)

Key Words: myocyte apoptosis and necrosis ■ myocyte regeneration ■ stem cells

This review will question the notion that cardiac hypertrophy results exclusively from enlargement of preexisting myocytes, which, in turn, are responsible for the initial adaptation and subsequent deterioration of the overloaded heart. This belief has been based on the generally accepted twin notions that (1) in the adult heart all myocytes are terminally differentiated and, therefore, cannot be recalled into the cell cycle, and that (2) the myocardium lacks a stem cell population able to generate new myocytes. This postulated inability of the myocardium to form new myocytes starting in the early postnatal period has straightjacketed and limited cardiovascular research in conceptually significant ways. If no new myocytes can be developed during most of postnatal life, then it follows that all therapeutic interventions need to be oriented toward the preservation of the remaining myocytes. According to this point of view, in a given heart all the myocytes are as old as the individual, leading to the outlandish conclusion that each and every one of the myocytes of a 90-year-old person are at least 90 years old. For the same reason, myocyte death should be a very rare event if cardiac mass and a certain level of function have to be preserved throughout the lifespan of the individual. Thus, according to the accepted paradigm, cardiac homeostasis is very static because there is no myocyte renewal and it is dependent on the ability of the cardiac myocytes to be as long lived as the individual.

We will present an alternative point of view of the myocardium that is based on the premise that myocyte death and regeneration are part of the normal homeostasis of the heart. During normal cardiac growth to adulthood, in addition to myocyte hypertrophy, new myocyte generation predominates over myocyte death and contributes significantly to organ growth. In pathological conditions and in the course of normal aging, when this balance is altered and myocyte...
formation is overtaken by cell death, the number of ventricular myocytes decreases, myocyte hypertrophy becomes apparent, and with time, chronic heart failure supervenes. New data question the concept that changes in myocyte size and shape are the exclusive structural modifications that condition the remodeling of the diseased heart. It is now clear that the generation of new myocytes plays a crucial role in the myocardial response to ischemic and nonischemic injury. Moreover, new myocyte generation, together with myocyte apoptosis and necrosis are major determinants of the evolution of pressure and volume overload hypertrophy pointing to common mechanisms in the response of the heart to workload and damage. Similarly, myocyte death and regeneration are important components of myocardial aging. Failure of new myocyte generation and hypertrophy to compensate for extensive cell dropout may be the most relevant factor in the onset of heart failure in the elderly.

Myocyte death and regeneration are proposed in the present review as the foundations of a new paradigm that, by integrating an increasing body of new information, can better explain normal cardiac homeostasis as well as the effects of disease processes and age on the anatomy and hemodynamics of the heart. This alternative view uncovers new opportunities for research and development of novel therapies for ischemic and nonischemic cardiomyopathies. The evidence for stemlike cells as source of the cardiac regenerative potential and origin of cycling new myocytes will be discussed. The use of these putative resident cardiac stem cells, as well as multipotent cells from other tissues, will be proposed as an alternative treatment of the failing heart.

Myocardial infarction and cardiac aging have been extensively studied and constitute by far the predominant causes of heart failure in the Western world. Contradictory views have been advanced with respect to the myocyte population dynamics predominant in these two entities that justify a review of the outstanding issues in an attempt to search for a new consensus on the biology of the myocardium.

Ischemic Injury and Myocyte Death

The concept of the heart as a terminally differentiated organ excludes any significant turnover of myocytes in the normal and diseased heart. This is because even moderate rates of myocyte death, over time, would lead to the disappearance of myocardial mass. Not surprisingly, therefore, the existence of myocyte death has been controversial. There is still considerable disagreement on the rate and mechanism of myocyte death, but the application of sensitive molecular probes is slowly generating the consensus that myocyte death is a quantifiable parameter in the normal and pathological heart of both human and experimental animals. It is surprising that even the most conservative values indicate that, in absence of myocyte regeneration, the normal heart would lose most of its mass in a few decades and the senile and failing heart would disappear in a matter of several months to a few years.

Cell death can occur by three mechanisms: apoptosis, necrosis, or their combination. Apoptotic and necrotic cell death have different consequences on cardiac remodeling. Myocyte necrosis leads to an inflammatory reaction, vessel proliferation, macrophage infiltration, fibroblast activation, and ultimately, scar formation. Conversely, after apoptosis, the reparative process does not involve collagen accumulation and apoptotic bodies are removed by neighboring cells with no apparent changes in the morphology of the tissue. However, apoptosis can induce acute restructuring of the ventricular wall and depression of tension development of the myocardium. Thus, the distinction between apoptosis and necrosis is important for understanding the impact of cell death on the mechanical behavior and structural composition of the heart.

The mechanisms of cell death in the infarcted heart have been characterized in animals and humans. According to the common view, necrosis is the only or the main mechanism of myocyte death after infarction. In contrast, there is evidence that apoptosis precedes necrosis and constitutes the prevailing form of myocyte death (Figure 1A through 1C). Shortly after the ischemic event, apoptosis affects more than 80% and necrosis less than 20% of the myocytes in the ischemic zone. With time, the two types of cell death overlap, the reparative process is activated, and myocardial scarring develops.

Little information is available concerning the pathways modulating apoptotic cell death in the infarcted heart. The implication of p53 and p53-dependent genes in the onset of cell death has been excluded in vivo, but in vitro experiments documented their involvement. Depletion of high-energy phosphates inhibits the vacuolar ATPase, enhancing electrolyte imbalance in the cells. Thus, acidosis may initiate myocyte apoptosis after myocardial infarction (MI).

Shortly after coronary occlusion, single- and double-strand DNA breaks are scattered throughout the surviving myocardium, although they are more numerous in myocytes located in the region adjacent to the dead tissue. Double-strand DNA breaks are consistently found in association with single strand breaks and apoptosis (Figures 1D through 1F). Single-strand breaks, however, are present in apparently healthy cells, suggesting that the mechanical overload of the spared myocardium triggered by the noncontractile ischemic area induces first repairable DNA damage and only subsequently irreversible injury and apoptosis. This form of cell death promotes a reorganization of the cells in the ventricle resulting in decreased wall thickness and increased chamber volume, primarily through side-to-side slippage of myocytes within the wall. Myocyte slippage can also occur chronically and, together with cell lengthening, contributes to the anatomical modifications of end-stage ischemic cardiomyopathy. In the terminal evolution of the disease, cell death is enhanced and occurs by apoptosis and necrosis.

In contrast to the mechanisms of myocyte death in the infarcted myocardium, p53 and p53-dependent and regulated genes play a critical role in the acute adaptation of the nonischemic portion of the heart. Myocyte stretch induced by diastolic dysfunction promotes the release of angiotensin II (Figures 1G and 1H) and the activation of AT1 receptors. Receptor activation leads to phosphorylation of p38-MAP kinase that, in turn, phosphorylates the C-terminal of p53 at Ser390. Because p53 DNA binding sites are present in the promoter of angiotensinogen and AT1 receptor, p53 enhances...
the myocyte renin-angiotensin system (RAS) and the formation of angiotensin II. In addition, p53 downregulates Bcl-2 and upregulates Bax. A decreased Bcl-2-to-Bax protein ratio makes myocytes more susceptible to death signals transmitted by angiotensin II.

AT₁-mediated formation of diacylglycerol (DAG) and inositol-3-phosphate (IP₃) in myocytes further enhances the activation of the death pathway. IP₃ mobilizes Ca²⁺ from intracellular stores and DAG is the physiological activator of PKC. PKC translocation to the plasmamembrane results in the phosphorylation of L-type Ca²⁺ channels increasing cytosolic Ca²⁺ that activates Ca²⁺-dependent DNase. However, the distal event of the cell death pathway is an increase in oxidative stress and DNA damage. In vitro experiments mimicking the effects of infarction on the surviving myocardium are characterized by the generation of reactive oxygen species, apoptotic cell death, restructuring of the muscle, and side-to-side slippage of myocytes. Mechanical deformation of adult ventricular myocytes in culture has a similar impact on RAS, p53, Bax, Bcl-2, and apoptotic cell death (Figure 2).

In summary, occlusion of a major coronary artery leads first to apoptotic myocyte death and, subsequently, to cell necrosis. Diastolic stretch of the surviving myocardium results in the release of angiotensin II and upregulation of the local RAS via activation of p53-regulated genes. The expression of p53-dependent genes and the formation of angiotensin II with stimulation of the AT₁ effector pathway promote single- and double-strand DNA cleavage, myocyte apoptosis, cell slippage, wall thinning, and chamber dilation. These anatomical changes contribute to the progressive impairment in function of the ischemic heart.
The finding that most myocytes irreversibly withdraw from the cell cycle soon after birth \(^{21}\) and the failure to develop a cell culture system capable of supporting myocyte replication further reinforced the notion that all adult cardiac myocytes are terminally differentiated. In addition, because in many species cardiac myocytes can become multinucleated and polyploid, \(^{20}\) the sporadic reports of DNA synthesis have been considered insufficient proof of true myocyte proliferation and it has made possible to dismiss the documented mitoses as examples of multinucleation with karyokinesis without cytokinesis. \(^{26}\)

The strongest argument in favor of new myocyte formation in the adult heart is the increase in myocyte number from birth to young adulthood in both animals and humans. \(^{27}\) These data, obtained by well-validated morphometric methods, \(^{27}\) are internally consistent because, in addition to demonstrating an increase in myocyte number, they show that the increase in cardiac mass during normal growth cannot be accounted for solely by myocyte hypertrophy. Moreover, the increase in myocyte number in the adult represents an underestimation of the actual number of myocytes formed due to the concurrent cell death with maturation. \(^{27}\) These data strongly suggest that myocyte renewal occurs throughout life in the myocardium and it is part and parcel of cardiac homeostasis. Interestingly, the renewal rate increases significantly under a variety of pathological conditions characterized mainly by an increase in cardiac wall stress. \(^{20,27}\)

The increase in myocyte number, as detected by morphometric analysis, does not provide information about the origin of these new myocytes. They could originate through the commitment of precursor cells to the myocyte lineage, by replication of preexisting myocytes, or by a combination of these two mechanisms. Because the concept of a precursor cell capable of differentiating into a myocyte in the adult heart is of recent origin, \(^{25,28}\) most of the controversy about myocyte growth and regeneration has concentrated on the evidence for or against the replication of existing myocytes. Unfortunately, this focus has been the source of many of the misinterpretations that still hamper this area of research.

In the 1960s, Linzbach was the first to notice an increase in myocyte number in severely hypertrophic human left ventricles. \(^{28a}\) A few years later, Rumyantsev \(^{29}\) reported that a limited percentage of adult ventricular myocytes can overcome the block in DNA synthesis and pass through all phases of the cell cycle. In the 1990s, work mainly from this laboratory \(^{20}\) confirmed these earlier observations, extended them to the whole myocardium, and provided quantitative measurements on the frequency of myocyte replication using a variety of cytological and biochemical markers, including PCNA, Ki67, and BrdU labeling. In addition, the myocyte mitotic index was determined in normal hearts and in acute and chronic cardiac failure of ischemic and nonischemic origin in mice, \(^{30}\) dogs, \(^{31}\) and humans. \(^{23,24,27}\) The high frequency of cardiac myocyte replication pointed to myocyte regeneration as an important contributor to the maintenance of cardiac mass under physiological and pathological conditions.

Unfortunately, neither nuclear proteins capable of identifying cells at the G1-S boundary (PCNA) \(^{24}\) and within the...
cell cycle (Ki67), nor techniques detecting replicating DNA, provide an actual quantitative measurement of the extent of myocyte proliferation. This is because there is no information on the duration of the cell cycle in fetal, neonatal, or adult myocytes in vitro or in vivo. Nevertheless, some approximate values can be indirectly obtained. The proportion of myocytes expressing Ki67 in vivo corresponds to the number of myocytes within the cell cycle at a specific time point (Figures 3A and 3C). There are no examples of Ki67-positive cells that are not in the cell cycle. These data and the number of myocytes in mitosis (Figures 3B and 3C) provide a good but approximate determination of the length of the myocyte cell cycle in the normal and decompensated heart. Because the duration of mitosis is \( \approx 30 \text{ minutes} \) and there are \( \approx 50 \)-fold more Ki67-positive myocytes than mitoses, a replicating myocyte should complete the cell cycle in approximately 25 hours. The interpretation of PCNA, BrdU, or \([^{3}H]\) thymidine labeling is complicated by the difficulty to distinguish true cell cycle replication from DNA repair. This limitation does not apply to Ki67. However, Ki67 may not distinguish between multinucleation and true myocyte division.

It has been argued that some of the DNA synthesis and mitotic figures detected in myocytes could represent an increase in binucleation instead of cytokinesis. Although this point of view is formally correct, two sets of data argue against it. First, the identification of myocytes undergoing true cytokinesis. Second, the lack of any increase in the population of multinucleated myocytes after puberty in all species analyzed, including man. This is noteworthy because if a significant fraction of DNA-replicating myocytes underwent karyokinesis without cytokinesis, the frequency of multinucleated myocytes should have increased dramatically with age. This is not the case.

When most of these observations were reported in the mid-1990s, in the absence of markers capable of distinguishing replicating from nonreplicating myocytes, this laboratory interpreted the evidence for myocyte division as an indication that not all myocytes had reached terminal differentiation. Clearly, the data documented that there were myocytes able to reenter the cell cycle with a probability that increased significantly in certain pathological conditions. Although we still believe that this interpretation is correct, we created confusion when we failed to distinguish that the capacity to reenter the cell cycle was limited to a small fraction of myocytes, whereas the remainder majority were terminally differentiated cells irreversibly withdrawn from the cell cycle. Because of the inability to distinguish between these two populations, instead of concentrating on the nature, origin, and destiny of the cycling myocytes, the arguments in the field have centered on whether or not all adult myocytes are terminally differentiated. The relevant issue is whether the adult heart has the capacity for new myocyte formation and, if so, its extent and biological significance. The arguments in favor of new myocyte formation in the adult heart are difficult to dismiss. Measurements of Ki67 and mitotic index have identified very high levels of myocyte proliferation in the viable myocardium acutely after infarction in humans. Indisputable evidence of large number of myocytes in mitosis and evidence of cytokinesis have been found, providing an unsuspected picture of the regenerative capacity of the heart.

A normal adult human left ventricle contains \( \approx 5.5 \times 10^9 \) myocytes and an infarct of 30% would decrease their number to \( \approx 3.8 \times 10^9 \). A mitotic index of \( 11/10^6 \) myocytes in the intact ventricle and an average \( 520/10^6 \) myocytes in the infarcted ventricle imply that at the sampling time there are 60,500 and 1,976,000 myocytes in mitosis in the normal and injured left ventricle, respectively. If this degree of proliferation measured at 7 days after coronary artery occlusion would persist, the \( 1.7 \times 10^9 \) myocytes lost with infarction would be replaced completely in less than 3 weeks. Although the mitotic index decreases to \( 150/10^6 \) myocytes in end-stage cardiac failure, the entire left ventricle could be replaced in
less than 6 months. These degrees of cell proliferation strongly suggest that the generation of new myocytes is a major determinant of ventricular remodeling. Myocyte hypertrophy with cell lengthening contributes to the process but is only one of the factors involved in ventricular dilation and wall restructuring. Growth within the viable tissue restores the original amount of cardiac mass, but it does not invade and substitute the necrotic or scarred ventricular wall.

In summary, a segmental loss of myocardium due to ischemic heart disease activates myocyte regeneration and hypertrophy that together contribute to the formation of new muscle mass. However, both growth processes are restricted to the remaining viable myocardium and the border zone. A scar develops and there are no therapeutic or surgical interventions at present capable of replacing the healed infarcted area with viable functioning myocardium.

### Pressure-Volume Overload and Myocyte Growth

The magnitude of myocyte replication that occurs in the overloaded heart in the absence of coronary artery disease can be accurately determined by the absolute increase in cell number in the ventricular myocardium. This fact, by itself, constitutes an incontrovertible demonstration of new myocyte formation in the adult heart. By this approach, myocyte proliferation has been demonstrated in animal models and humans with cardiac failure. Increases in cell number up to 60% or more have been identified, indicating that the mammalian heart possesses a significant growth reserve and a large number of new myocytes can be formed in a relatively short time. More recently, we have identified very high levels of myocyte regeneration in patients with aortic stenosis.27,35

However, myocyte death by apoptosis and necrosis is inevitably present in the overloaded heart. This phenomenon complicates the estimation of the real amount of newly formed cells by any methodological procedure. Myocyte hyperplasia is underestimated by cell death and cell death is underestimated by myocyte regeneration.27

The underlying hemodynamic condition plays a major role in the growth response of myocytes in the overloaded heart with no signs of ischemic injury. If cardiac dysfunction is not present and the ventricle is hemodynamically compensated, myocyte hypertrophy is the predominant form of cell growth, and myocyte proliferation is not significantly above that in control hearts. Conversely, myocyte multiplication becomes the major growth adaptation of the failing heart. These relationships have been observed in humans and animals exposed to pressure overload. Little information is available in volume overload-induced cardiac hypertrophy, but some results support the conclusions reached for pressure overload.

In summary, absolute increases in myocyte number have been documented in the heart after pressure and volume overload hypertrophy. The underlying hemodynamic condition, like the state of coronary perfusion, appears to influence the growth response of the myocardium. Ventricular dysfunction and failure associated with the activation of new myocyte formation, whereas myocyte hypertrophy characteristically is more pronounced in the functionally compensated ventricle. Thus, the old concept that myocyte division is triggered only after maximal cellular hypertrophy is reached should be reconsidered. Results in the aging heart further challenge the heart weight theory based on the hypothesis that cell replication is secondary to extreme myocyte enlargement.

### Myocyte Aging: Cell Growth and Cell Death

The process of aging offers an extraordinary example of the effects that the changing balance between cell death and cell growth has on the pathological restructuring of the heart. The accepted paradigm claims that the number of ventricular myocytes is established at birth and these cells contract and maintain cardiac function until death of the organism. However, it is now clear that myocytes undergo continuous turnover and dying cells are constantly replaced by newly formed myocytes. In the aging heart, a subpopulation of myocytes undergoes DNA replication and mitosis, another subpopulation undergoes hypertrophy, and yet another group experiences apoptosis and necrosis. There is no doubt that myocyte death occurs throughout the lifespan of the organism independently from cardiac diseases. In the normal heart, the rate of cell death increases with age and after middle age it is not balanced by a concomitant increase in new myocyte formation. The excess cell death results in a net reduction in myocyte number. This smaller number of viable myocytes hypertrophies to preserve myocardial mass resulting in an old heart of normal or slightly decreased size but with enlarged parenchymal cells. Thus, myocyte death, hypertrophy, and new myocyte formation characterize the aging heart.

The recognition that myocytes are continuously replaced in adulthood and senescence, and that cell regeneration is enhanced by hemodynamic overloads and ischemia, indicates that cells of different ages are present during the entire life of the nondiseased and diseased heart. These distinct cell populations differ in their ability to react to growth stimuli. The history of a cell conditions the type and magnitude of its growth response and its capacity to succumb to or survive apoptotic and necrotic death signals. Whether a myocyte’s response to increased load is replication, hypertrophy, or cell death is largely influenced by its size, which, in turn, reflects the age of the cell. Large myocytes are old, do not react to growth stimuli and are more prone to activate the cell death pathway. Smaller cells are younger, possess the ability to hypertrophy and are less susceptible to cell death. The smallest cells have been born only recently and still can undergo a limited number of cell cycles.

When ventricular myocytes of 4-month-old rats are separated according to their dimension by gravity sedimentation in isotonic Percoll solution, they range in size from 4000 μm² to 110 000 μm² (Figures 4A through 4G). In almost all cases, myocytes 90 000 μm² in volume and larger are positive for p16^Nκ4a (Figure 4H), a protein that inhibits the reentry into the cell cycle; p16^Nκ4a is a marker of cellular aging. Apoptosis is much higher in these larger cells that express p16^Nκ4a, whereas myocytes 15 000 μm² in volume and smaller rarely express p16^Nκ4a and have low rate of apoptosis (Figures 4A through 4H). Thus, cell death is correlated with the size of the...
cell and with the distribution and level of this marker of cellular senescence.

The inability of old myocytes to hypertrophy and/or replicate becomes apparent when myocardial infarction is induced in young adult animals and the replicating myocytes are BrdU-labeled before euthanasia. At 7 days after infarction, DNA replication is restricted to small cells and decreases progressively with increasing cell size (Figure 4I). There is an almost perfect antithetic relationship between cell replication and p16INK4A expression. p16INK4A positive cells do not reenter the cell cycle, and cycling cells do not express p16INK4A. Neither hypertrophy nor replication occurs in very large myocytes. Myocytes 35,000 μm³ in volume and larger do not reenter the cell cycle and their growth response is limited to cellular hypertrophy. The fraction of myocytes with extreme dimensions and impaired growth increases with age, progressively affecting the response of the old heart to pathological stimuli. The age-dependent increase in myocyte death, coupled with reduction in the coronary vasculature, further deteriorates the functional adaptation of the senescent heart. These phenomena may explain why coronary heart disease and its complications are major risk factors in the elderly, and myocardial infarction is associated with increased morbidity and mortality in this population.
In summary, the adult heart should not be considered a postmitotic organ. The myocardium exhibits a significant regenerative capacity exemplified by a subpopulation of cycling myocytes whose presence throughout life continuously changes the proportion of young and old cells in the heart. Small myocytes are young and retain the ability to divide and enlarge. Large myocytes lose the potential for cellular hypertrophy and proliferation and these defects increase with age. Large cells express more inhibitors of the cell cycle and, in response to work overload, are unable to activate the program responsible for the quantitative and qualitative changes in gene expression characteristic of myocyte hypertrophy. Relative cell size may be viewed as an indicator of myocyte age, whereas the accumulation of large myocytes characterizes organ aging.

**Bone Marrow–Derived Cells Are Able to Reconstitute the Myocardium**

The regenerative properties of the myocardium, although substantial in most cases, fail to reconstitute the myocardium lost after coronary artery occlusion. Over the past few years, a flurry of attempts have been made in animal models and even in humans to implement protocols of cell therapy aimed at replacing the lost myocytes with autologous or heterologous contractile cells injected into or around the area of necrosis. The two most favored donor cell types have been skeletal myoblasts and embryonic or neonatal cardiomyocytes. Although many of the cell transplantation experiments reported improvement in cardiac function, it has been difficult to demonstrate long-term benefit as well as integration and electrical coupling of the transplanted cells with the host myocardium. Not surprisingly, the inability of skeletal myoblasts and embryonic or neonatal cardiomyocytes to transdifferentiate into cardiocytes and form gap junctions with the host myocytes has been a serious shortcoming and probably it has been responsible for arrhythmias due to reentry mechanisms. The use of primary fetal or neonatal cardiac myocytes or cell lines derived from them or from embryonic stem cells has got around this problem. However, if extrapolated to the human, in addition to the difficulty in obtaining sufficient cells to make a functional difference, this approach would require the use of heterologous cells with all the attendant problems of histocompatibility and immunosuppression. It is not an exaggeration to conclude that these cells have proven to be far from the ideal donors. The ideal candidate donor cell for myocardial reconstitution is an autologous cell that can be easily obtained and that, once placed into the myocardium, homes to the lesion, has a robust replication capacity with a low risk of neoplastic transformation, and differentiates into morphologically, biochemically, and functionally normal cardiocytes (see Figures 5A and 5B).

An increasing number of reports have recently documented that most adult mammalian tissues contain a population of multipotent undifferentiated cells with many of the characteristics of stem cells. These cells, under the appropriate conditions, are able to reconstitute many or all the cell types of the tissue of origin. Surprisingly, several of these putative stem cells exhibited an unsuspected degree of plasticity and were shown to be able to differentiate in cell types other than those in the tissue of origin. Although the molecular mechanism(s) responsible for determining the developmental choice of these stem cells remains to be elucidated, it soon became clear that damage of the host tissue was a potent stimulus to coach these cells to adopt the fate of the host organ. In the absence of tissue damage, most of the introduced cells either disappeared rapidly or did not expand and integrate into the host organ. The cell type with the highest degree of plasticity and differentiation potential was the hematopoietic stem cell (HSC). These cells were able to differentiate into neurons, glia, hepatocytes, and skeletal muscle. These reports together with the known overlap between the hematopoietic and cardiac developmental programs prompted us to test whether bone marrow–derived cells (BMCs) injected in the border zone of a recent myocardial infarction would transdifferentiate into cardiomyocytes.

A population of lineage negative BMCs enriched for the expression of c-kit, the receptor for stem cell factor (SCF), were injected into the border zone of a recent infarction. The
implanted BMCs amplified many folds and induced myocardial regeneration that extended to the whole area of necrosis. The newly generated ventricular wall was composed of small myocytes with well-defined sarcomeres and positive for all the cytoplasmic and nuclear cardiocyte markers analyzed, including connexin 43. More unexpected, this new myocardium had an abundant network of donor-derived functional coronary vessels and capillaries. The formed myocardium contracted synchronously with the rest of the ventricle and improved cardiac function.56 Thus, BMCs were able to generate the three major cell types of the heart: myocytes, vascular smooth muscle and endothelial cells. Unfortunately, because the injected cells were heterogeneous, it was not possible to ascertain whether the developing myocardium was produced by single or multiple donor cell types, and whether myocytes, smooth muscle, and endothelial cells originated from the same or different precursors. The results, however, unambiguously demonstrated that the bone marrow contains cells that are able to reconstitute a functional, albeit immature, vascularized myocardium. One of the striking characteristics was the absence of donor cells or their progeny within the nonischemic host myocardium. The administrated cells had selectively homed and expanded into the infarcted area, suggesting the existence of a strong chemotactic agent that attracted them to the damaged zone where they became activated, highly proliferative, and induced to adopt the cardiac phenotype.

Systemic administration of cytokines, such as SCF and granulocyte-colony stimulating factor (G-CSF), mobilizes pluripotent cells from the bone marrow, raising their level in the peripheral circulation by many fold.57 Thus, we hypothesized that BMCs mobilized by SCF and G-CSF might be able to home to infarcted myocardium, transdifferentiate, replicate, and regenerate the lost myocardium without the need of harvesting and injecting them into the myocardium.

Using an infarct model similar to that used for the injected BMCs, we showed that cytokine mediated activation of pluripotent bone marrow cells around the time of coronary occlusion resulted in highly efficient myocardial regeneration four weeks later. This regeneration decreased mortality, infarct size, cavity dilation, wall thinning and diastolic wall stress. Ejection fraction increased and hemodynamics improved as the result of the formation of ~15×10⁶ new myocytes accompanied by new arterioles and capillaries connected to the primary coronary circulation.57 These results demonstrate that the cytokine regime was able to mobilize and activate multipotent cells with the capacity to home to the damaged myocardium, undergo significant expansion, differentiate into the three main cardiac cell types, and organize into an anatomical and functional myocardium. However, because we did not use animals with a genetically tagged bone marrow, these results fell short of formally proving that the regenerating cells originated from the bone marrow. In fact, they could have originated from any other organ of the body or even from the spared portion of the myocardium. Nevertheless, the known effect of the cytokines and the extensive use of similar protocols to stimulate the bone marrow in humans, make this tissue the most likely source of the myocardium regenerating cells.

Recently, several reports have cast doubts about the transdifferentiation potential of HSCs, because of failure to reproduce some of the results, demonstrating their multipotentiality.58 The hypothesis has been put forward that the donor stem cells might fuse with the parenchymal cells of the host tissue. This would give the appearance of transdifferentiation when in reality the expression of the host tissue differentiated phenotype is limited to the heterokaryons formed.59,60

Our results of myocardial regeneration with BMCs and cytokine-mobilized cells do not address the issue of HSCs plasticity and transdifferentiation potential because we do not have evidence that the myocardium-regenerating cells are the HSCs. Our data only documents the existence of myocardium-regenerating cells in the bone marrow56 and among the cells susceptible to be mobilized by cytokines.57 The group of Verfaillie61 has identified in the bone marrow multipotent adult progenitor cells (MAPCs) that differentiate into a variety of cell types, including cardiocytes. We do not know the relationship, if any, between these stromal cells and the c-kitPOS BMCs we injected or between them and the cytokine-mobilized cells. It is noteworthy that the MAPCs are c-kit negative, whereas ours were c-kitPOS. Although it is possible that contaminant c-kit–negative cells in our preparation were responsible for the myocardial regeneration, it seems highly unlikely given the small number of injected cells and the very large number of new cardiac cells formed.56,57 The data presently available cannot exclude that the MAPCs and our cells represent two different differentiation stages of the same cell type.

Five lines of evidence argue against cell fusion as the cause for cardiac phenotype and pattern of gene expression of the injected and mobilized cells. (1) In our model of infarct there are extremely few host myocytes left in the necrotic zone. It is unlikely the donor cells would readily find a host fusion partner. (2) There are no detectable donor cells in the spared myocardium where they would be expected to find ready fusion partners. (3) The vast majority of newly formed myocytes have a diploid DNA content. (4) The newly formed myocytes have an average volume of 750 µm³, whereas the host myocytes are 20- to 25-fold larger. (5) The donor-derived cells divide rapidly and extensively while, in general, tetraploid cells divide slowly and might not divide at all if one of the partners is a terminally differentiated cardiac cell.

Therefore, we believe that our results document the existence of bone marrow and cytokine-mobilized cells able to differentiate into bona fide cardiac cells and coronary vessels. The precise identity of the cells with this potential remains to be established.

**Myocardial Cell Formation From Stem-Like Cells in the Adult Heart**

The cytokine administration regime used for BMC mobilization produces high levels of circulating bone marrow–derived multipotent cells.57 This is the amplification of a naturally occurring phenomenon because under normal conditions there are bone marrow–derived stem cells in the circulation.57 Is it possible that these circulating cells continuously colonize the myocardium and contribute to myocyte renewal? The
cases of sex mismatched cardiac transplants in humans where a female heart is transplanted into a male host offered an ideal setting to test this hypothesis. The colonization and differentiation of host cells homed to the transplanted heart can be identified by the presence of the Y chromosome in the host-derived cells. We and others have shown that soon after transplantation, the female hearts had a significant number of Y chromosome positive myocytes and coronary vessels. Although there are discrepancies in the amount of detected chimerism among different groups, most likely due to technical differences, there is broad agreement on the nature and interpretation of the phenomenon.

Undoubtedly, these male cells originated from host cells that colonized the transplanted heart and subsequently differentiated. These differentiated host cells in the donor heart are an irrefutable proof of new myocyte formation in the adult heart and presuppose the existence of mobile stem-like cells able to differentiate into the three main cardiac cell types. It is unlikely that this mechanism of generating cardiocytes, coronary arterioles, and capillaries is restricted to cardiac transplantation. It is more reasonable to assume that this colonization represents a normal homeostatic process for the maintenance of the myocyte population and the vasculature that the sex mismatch of these patients permitted it to be identified and quantitated.

The significant numbers of Y chromosome–positive cardiomyocytes in the donor female heart presupposes the colonization of the transplant by a precursor cell capable of adopting the cardiogenic phenotype. It is not credible that migrating host cardiomyocytes colonized the donor female heart. Moreover, these Y chromosome–bearing cells in the female heart are not the result of cell fusion of host cells with donor cardiomyocytes because they contain a single X chromosome. We hypothesized that these migrating myocyte-generating cells were likely to have many of the characteristics of stem or precursor cells that should make them identifiable in the transplanted heart myocardium. Primitive cells of donor and recipient origin that express stem cell–related surface antigens were identified in the donor hearts and in the atrial stumps of the recipients. Most importantly, identical cells were found in control hearts. These primitive cells were detected by three surface markers commonly expressed in stem cells: c-kit, MDR1, and an epitope related to stem cell antigen-1 (Sca-1). Additionally, cardiac progenitors and precursor cells of male and female origin were recognized by the coexpression of the stem cell antigens and transcription factors of the myocyte lineage or receptors typical of endothelial cells and smooth muscle cells. Together, these findings point to the existence of stem-like cells with the potential of regenerating the components of the myocardium: muscle cells and coronary vasculature. The data did not offer information on whether all cell types originated from one or several different precursors.

Another unanswered question is the origin of these cardiac stem-like cells. Because cardiac transplants result in chimeric hearts with recipient and donor tissue, two sources for these cells are possible: (1) the remnants of the recipient heart in which an atrial stump, containing the outflow of the venae cavae is sutured to the donor heart; (2) circulating cells that home to the transplanted heart. It is an issue of biological and clinical significance to determine whether the cardiac stem-like cells are resident cells that accumulated in the heart early in development or whether they are the progeny of stem cells that, throughout life, home to the myocardium through the systemic circulation.

Cardiac Cells With Stem Cell Characteristics
Whatever the origin of the primitive cells discussed, their presence in the normal and transplanted heart, together with the identification of some of them after having initiated the gene expression program of one of the cardiac cell lineages, is suggestive that they might be true cardiac stem cells. This evidence, however, still falls short of establishing a precursor-product relationship between them and the fully differentiated cardiac cells. To address this issue, we tested whether the c-kit cells found in the adult heart behave like true adult cardiac stem cells. Un differentiated Lin-c-kit cells were isolated from the ventricle of adult rats (see Figure 5C), and after plating, these cells were cloned. The progeny of single cells expressed biochemical markers of myocytes, smooth muscle, and endothelial cells. These cells were able to acquire a structurally fully differentiated phenotype when placed in a damaged myocardium (unpublished data). This result suggested that the cloned cells were multipotent and that myocytes, smooth muscle, and endothelial cells originated from the same adult precursor. Whether or not these cells fulfill all the criteria that some believe are required before the label of “stemness” can be applied to a given cell type is beside the point. Independently of what is the appropriate name for these cells, the evidence is convincing that the progeny of a single cell is able to produce the three main cardiac cell types. However, the presence of cells with cardiac stem cell properties in the myocardium conclusively dispels the notion that the heart is a terminally differentiated organ without self-renewal potential and provides an explanation, as well as a conceptual context, for the existence of cycling myocytes.

The presence of stem-like cells in the normal and pathological myocardium provides a new interpretation for the presence of cycling ventricular myocytes. It is now clear that these cycling myocytes are the progeny of primitive cells recently differentiated into the myogenic lineage that have not yet reached their terminally differentiated state. These cycling myocytes are a transient population with relatively limited proliferative capacity in a state of constant renewal. New cycling cells may constantly enter this pool through the differentiation of the stem-like cells, whereas other myocytes continuously exit this pool when they become terminally differentiated, losing their capacity to reenter the cell cycle. The kinetics of this pathway is influenced by physiological and pathological parameters. Ischemic heart disease and cardiac failure increase the rate of flux through this transient pool. Activation and proliferation of the stem-like cells leads to an increase of new myocyte formation to compensate for the increased myocyte death that characterizes these entities.

The transient nature of the replicating myocyte pool raises the question of its reliability as a marker for new myocyte
generation. Without information on the rate of cell entry into exit from this pool and the number of replications that these cells can undergo, quantification of the actual number of cycling myocytes does not provide any more information about the rate of new myocyte formation than the number of reticulocytes in peripheral blood provides about erythropoiesis. Yet, in the absence of more accurate indicators it is an indirect estimate of the parameter of interest. The physiologically meaningful parameter, however, is the rate of new myocyte formation of which the number of cycling myocytes is only an indirect indicator.

Although we have not yet found distinctive markers that permit a priori identification of newly born myocytes that are still able to replicate, there are some characteristics prevalent among them. The dividing myocytes are significantly smaller than the mature myocyte and their peripheral sarcomeric organization resembles that of fetal or neonatal myocytes. They are all p16INK4a negative, although the majority expresses in their nuclei the catalytic subunit of telomerase and Ki67 protein. The antiapoptotic function of telomerase in presses in their nuclei the catalytic subunit of telomerase and functions. Conversely, it cannot be excluded that telomerase exerts an antiapoptotic action in cells capable of dividing and, thereby, plays a critical function in preserving the growth reserve of the adult normal and diseased heart.

In summary, primitive cells with properties of stem cells are present in the myocardium, either as a resident population of embryonic origin or as a blood-borne population that continuously seeds the tissue. This arrangement points to a mechanism for the continuous renewal of myocytes and coronary vessels throughout the lifespan of an individual. Additionally, it provides the basis for an increase in myocardial mass in response to physiological or pathological demands that impose an enhanced load on the heart. Clearly, more work is needed to gain a better understanding of the identity, lineage, and biology of these cells as well as the regulatory network responsible for their multiplication and differentiation.

The adult heart has a significant capacity for myocyte regeneration that is markedly enhanced in acute and chronic heart failure of ischemic and nonischemic origin. We hope this new view of myocardial growth and homeostasis will lead to a broadening of the long-term research and therapeutic goals for the diseased heart. Understanding the regulation of new myocyte formation will provide not only a better understanding of cardiac homeostasis but will also open new avenues for therapeutic intervention. These new approaches based on harnessing the self-renewal potential of the myocardium should improve the chances to produce true myocardiogenic regeneration that will maintain or restore cardiac function and reduce the need for cardiac transplantation.

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References


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