Sequestration of p27 within the cytoplasm of cardiac myocytes in chronic ischemic heart disease: pathogenic implications for ischemic cardiomyopathy

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Abstract A number of recent studies have suggested that cardiac myocytes, previously considered post-mitotic, re-enter the cell cycle and possess the ability to proliferate with certain pathogenic stimuli. To study this further, we examined cellular proliferation in myocardial tissue from subjects with chronic ischemic heart disease-associated myocardial infarction and subsequent congestive heart failure. We found striking increases in cytoplasmic phospho-p27, a well-known mitotic regulator, compared to controls by both immunocytochemical and immunoblot analyses. However, we found no evidence for cardiac myocyte proliferation in either disease or control subjects using both mitotic counting (no mitotic figures were observed) and Ki-67 immu-

nocytochemistry, which demonstrated a 0% proliferation index. That increased cytoplasmic phospho-p27 is not accompanied by division prompts us to speculate that ectopic cell cycle activation occurs in the face of minimal to absent myocyte proliferation per se. Based on these findings, and the parallel findings in post-mitotic neurons in neurodegenerative disease, we suggest that cell-cycle activation in ischemic heart disease is a deleterious event that perpetuates disease pathogenesis culminating in myocardial failure.

Key words binucleation · cell cycle · immunocytochemistry · Ki-67 · mitosis · myocardial infarct · myocyte · p27 · proliferation

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Introduction

Cardiac myocytes withdraw from the cell cycle soon after birth, and have been traditionally considered post-mitotic (Ueno et al. 1988). However, recent studies have suggested that the adult myocardium is capable of limited cell division in certain pathological conditions (Kajstura et al. 1998; Beltrami et al. 2001). Heart regions adjacent to myocardial infarct, for instance, showed a Ki-67 proliferation index of 4%, while a 1% index was noted distant from the lesion. The same study demonstrated mitotic indices of 0.08% and 0.03% respectively. In an earlier

study, Kajstura and colleagues (1998) found that 14 myocytes per million were in mitosis in control human hearts, with a 10-fold increase in end-stage ischemic heart disease (152 myocytes per million) and in idiopathic dilated cardiomyopathy (131 myocytes per million). They further showed changes consistent with cytokinesis, and suggested this finding as “unequivocal” evidence of cell division in adult cardiac myocytes. These fundamental studies are supported in part by a number of biochemically more specific studies suggesting cell-cycle activation in disease. For example, Tamamori-Adachi et al. (2003) suggested that post-mitotic cardiomyocytes could be stimulated to proliferate with nuclear import of cyclin D1, while Sano and colleagues (2002) showed that Cdk-9 was limiting for cardiac growth.

The evidence that cardiac myocytes retain some capacity to enter the cell cycle has provided reason for optimism in terms of exploiting proliferative potential for therapeutic purposes, such as through adenovirus-mediated gene transfer (Agah et al. 1997). The mechanistic basis for such a strategy is substantiated by evidence of apoptosis, and enhanced programmed cell death in the face of increased Bcl2 expression, in heart failure (Narula et al. 1996; Olivetti et al. 1997), i.e., it suggests that cardiac decompensation is due to terminal apoptosis, and therefore that enhanced cardiac myocyte proliferation in this setting may prolong meaningful cardiac function. However, it is unclear whether the simple addition of contractile elements in cardiac failure, through cell cycle activation, and subsequent hypertrophy/hyperplasia of cardiac myocytes, would lead to functional improvement or alternatively would exacerbate cardiac decompensation.

To address the issue of whether myocyte proliferation is adaptive or deleterious, we examined heart tissue affected by previously documented ischemic heart disease (myocardial infarct) that was clinically associated with congestive heart failure for features of cell-cycle activation (mitotic activity, Ki-67 proliferation index), and phosphorylated p27 expression. The p27 CDK inhibitor plays an essential role in transition through the G1 phase, in particular the restriction point, via binding to and inhibiting cyclin-CDK complex in the nucleus (Reynisdottir

et al. 1995). The phosphorylation of p27 promotes its cytoplasmic localization and degradation (Sutterluty et al. 1999; Blain and Massague 2002). Aberrant cytoplasmic expression of phosphorylated p27 has recently been shown in malignant neoplasia as a feature of excessive and disordered cell-cycle activation (Liang et al. 2002). We hypothesized therefore that cytoplasmic expression of phosphorylated p27 might be increased in diseased hearts, and, as is the case with malignant neoplasia, may represent a maladaptive response to increased cardiac stress that exacerbates, rather than mitigates, cardiac decompensation.

Materials and methods

Cases Heart tissue was examined at autopsy from seven subjects with ischemic heart disease (ages 44–92; heart weights 500–630 grams) and four subjects with no evidence of ischemic heart disease (ages 43–73; heart weights 350–520 grams). All disease subjects suffered both ischemic heart disease (myocardial infarct) and congestive heart failure during life.

Frozen heart tissue from two control subjects and two diseased subjects were obtained from the University of Maryland Brain and Tissue Bank.

Histopathology Hearts examined at autopsy were fixed by immersion in 10% neutral buffered formalin, processed through serial ethanol and xylene solutions, and embedded in paraffin. Six- μ m-thick sections were prepared for routine histopathology and immunohistochemistry.

Mitotic Index Review of all available slides from diseased and control subjects for mitotic activity was performed. Counts are expressed in n per mm^2 .

Ki-67 Proliferation Index Evidence of cell-cycle activation using nuclear immunoreactivity of Ki67 (monoclonal Ki67, Ventana) was determined. Cells showing any degree of immunoreactivity were analyzed. Nuclei within the interstitium—e.g., fibroblasts, endothelial cells,

whether positive or negative—were excluded from the analysis. Counts were performed using manual counting. All nuclei present in the sections were evaluated.

Phospho-p27 Immunocytochemistry Sections were deparaffinized in xylene and then hydrated through graded ethanol to Tris buffered saline, pH 7.6 (TBS). Endogenous peroxidases were eliminated with 3% hydrogen peroxide in methanol for 30 minutes. Non-specific binding sites were blocked with 10% normal goat serum prior to the addition of the primary antibody. [Phospho-p27 (Thr187), 1:100 (Zymed)] was applied followed by rabbit secondaries, and visualized using the peroxidase anti-peroxidase method with 3,3'-diaminobenzide as the co-substrate (Sternberger 1986). The specificity of this antibody was previously demonstrated in our laboratory (Ogawa et al. 2003). Nuclear marker Ki-67 was used to demonstrate evidence of cell cycle activation and determine the proliferation index.

Microscopic images were obtained using an Olympus BX-41 light microscope and a Nikon DXM1200 digital camera with ACT1 software.

Immunoblot Analysis Tissues from diseased patients and control cases were homogenized in 10 vol. of TBS containing 0.02% Sodium azide, 0.5% Sodium deoxycholate, 0.1% SDS, 1% NP-40, 1 mM PMSF, 1 µg/ml aprotinin and 1 µg/ml antipain. Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (10 µg/lane) and electroblotted onto Immobilon-P (Millipore, Bedford, MA, USA) by standard procedures as previously described (Zhu et al. 2000). Transferred blots were incubated sequentially with blocking agent (10% non-fat milk in TBS-Tween), rabbit anti-phospho-p27 (1:200), or p27 antibody (1:500) and affinity-purified goat anti-rabbit immunoglobulin peroxidase conjugate pre-absorbed to eliminate human cross-reactivity (1:1500) (StressGen Biotechnologies Corporation, Inc., Victoria, B.C., Canada). Blots were developed by the ECL technique (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) according to the manufacturer's instruction. Blots were stripped in stripping

buffer (2% SDS, 62.5 mM Tris-HCl, 100 mM β-mercaptoethanol, pH 6.8) for 30 min at 60°C and then probed with antibody against actin (1:1000), which is constitutively expressed as a loading control.

Results

Histopathology

Routine histopathology of the ischemic heart disease cases demonstrated scarred myocardium accompanied by varying cardiac myocyte hypertrophy (increased contractile elements, enlarged

Figure 1 Immunocytochemistry for phosphorylated p27 in control subject (a) and subject with a remote myocardial infarct (b). The photomicrograph in a is taken from a transition zone between the infarct and the surrounding myocardium. Note the intense immunolabeling of cardiac myocytes in comparison with collagenous scar tissue (*pale areas*). In contrast, the control subject shows no significant reactivity. Scale bar = 50 µm

nuclei). The control hearts showed minimal variation in cardiac myocyte diameter, and no specific pathological changes including myocyte hypertrophy. The results were quantitated as indicated below. No inflammatory infiltrates were detected in either the disease or control subjects.

Immunocytochemistry and immunoblot analysis for p27

Phospho-p27 immunocytochemistry showed strong immunolabeling of cardiac myocytes, which was uniform throughout the myocardial tissue, being present in similar amounts in the vicinity of the myocardial infarcts and far removed from those areas (Figure 1). The immunoreactivity localized only to myocyte cytoplasm. No nuclear staining was observed. Nor did we observe staining of interstitial elements such as fibroblasts, endothelial cells, or vascular

Figure 2 Immunoblots of heart tissue from ischemic heart disease subject (*HD*) and control (*con*) probed with antibody against phospho-p27, demonstrating a band at the expected molecular weights of 27 kDa. The band is stronger in diseased homogenate compared to control. However, the band recognized by p27 antibody (total p27, phosphorylated and non-phosphorylated) is stronger in control tissue. Similar amounts of protein were loaded, as evidenced by actin blot

Figure 3 Ki-67 immunocytochemistry. No Ki-67-positive nuclei were detected in cardiac myocytes. An immunolabeled leukocyte within a capillary, serving as an internal control, is shown. Scale bar = 20 μ m

smooth muscle. In contrast, the tissue from control subjects showed only minimal background staining. No differences in phospho-p27 immunoreactivity as a function of post-mortem interval were noted in the diseased subjects. Immunoblot analysis revealed a stronger band at approximately 27 kDa in heart disease subjects compared to control (Figure 2), consistent with the immunocytochemical findings and further demonstrating antibody specificity. Interestingly, the p27 level (total p27, phosphorylated plus non-phosphorylated) was decreased in heart disease subjects compared to controls (Figure 2).

Cardiac myocyte proliferation

Mitotic Activity Review of all heart sections in both diseased subjects and controls was performed. In the sections analyzed, no mitotic figures were encountered, either in the myocardium or within the interstitium.

Ki-67 Proliferation Index The entire cross sections of all heart sections available for examination were analyzed. Neither the subjects with ischemic heart disease nor control subjects demonstrated Ki-67-positive myocytes. In a subset of both disease and control subjects, scattered Ki-67-positive nuclei were noted within the interstitium (Figure 3), interpreted as reactive/reparative mesenchymal elements.

Discussion

In this study, we demonstrate an increased p27 phosphorylation in the cytoplasm and a decreased p27 expression associated with cardiac myocytes affected by ischemic heart disease. Since p27 has a negative effect on cell proliferation via binding and inhibition of cyclin E/cdk2 in the nucleus and thus leads to G1 arrest (Reynisdottir et al. 1995), the phosphorylation and retention of p27 in the cytoplasm and decreased expression of total p27 may have a pro-mitogenic effect on cardiac muscle. This is consistent with the accumulating evidence of cell-cycle activation within cardiac myocytes in disease (Kajstura et al. 1998; Beltrami et al. 2001; Tamamori-Adachi et al. 2003). On the other hand, myocyte proliferation per se, based on Ki-67 immunolabeling and mitotic counting was entirely absent, suggesting aberrant cell-cycle processes without myocyte proliferation.

The serine/threonine protein kinase Akt/protein kinase B, which plays a pivotal role in tumorigenesis, has been shown to phosphorylate p27, resulting in p27 mislocalization in the cytoplasm (Viglietto et al. 2002). This may represent one mechanism for cytoplasmic p27 localization and cell-cycle activation in cardiac myocytes, as shown here. Not co-incidentally, our preliminary results indicate Akt is also activated in cardiac myocytes in diseased cases (Zhu, Smith and Castellani, unpublished data). It is also noteworthy that excessive phospho-p27 has been demonstrated in some tumors with aberrant cytoplasmic localization (Ciaparrone et al. 1998; Singh et al. 1998; Baldassarre et al. 1999). Notably, Skp2-containing E3 ubiquitin ligase, SCF, and its cofactor, CDK subunit 1 (Cks1), recognize Thr187-phosphorylated p27 and promote its degradation by proteasome (Sutterluty et al. 1999; Carrano et al. 1999). Therefore, phosphorylation of p27 also enhances its degradation, which is consistent with our finding that total p27 level is decreased in diseased cases where phospho-p27 is increased.

The precise effect of cell-cycle activation on cardiac pathophysiology is unclear. The presence of enlarged cardiac myocytes in hypertrophic and other cardiomyopathies indicates the addition of

contractile elements in non-dividing cells. Moreover, while the common finding of myocyte binucleation in cardiac muscle may suggest nuclear division without cytokinesis, studies to date, including the present study, have not shown an increased number of binucleated cardiac myocytes in disease (Olivetti et al. 1996). Therefore, the possibility that hypertrophy, rather than hyperplasia, characterizes the chronic cardiac disease state cannot be excluded. The reported low mitotic indices in tissues adjacent to and remote from infarcted heart tissue respectively (Beltrami et al. 2001), and a Ki-67 proliferation of 4% to 1% (a level comparable to anaplastic gliomas) (Tortosa et al. 2003), tend to refute the hypertrophy-only concept. Nevertheless, the mitotic counts are modest (expressed in numbers per million cells) and the corresponding studies provided no confirmatory evidence that the dividing cells were in fact cardiac myocytes and not reparative interstitial components. Moreover, in this study, we observed neither mitotic figures, nor Ki-67-positive myocyte nuclei in either disease or control subjects. We also found no differences in myocyte binucleation between disease and control subjects, suggesting that low-level cardiac myocyte binucleation is a physiological phenomenon. Taken together, these findings indicate that while cell-cycle activation is a feature of chronic ischemic heart disease, cell division per se is minimal to absent. Therefore, the increased cytoplasmic phospho-p27 immunolabeling and decreased p27 expression in chronic ischemic heart disease may well represent aberrant cell cycle activation that potentiates cellular dysfunction and overall myocardial failure; but at present there is no evidence that cell division is taking place in the overwhelming majority of these cardiac myocytes.

Hypertrophy of the myocardium is generally thought of as an adaptive or compensatory response to increased workload in a variety of pathological settings, from hypertension to valvular disease to congenital heart disease (Sadoshima and Izumo 1997). It should also be noted that increased ventricular mass is an adverse prognostic indicator in cardiovascular disease (Verdecchia et al. 2001). This therefore

suggests that blunting, rather than activating, the growth response might represent a useful strategy for developing new therapies for cardiomyopathies (Esposito et al. 2002). This strategy is in contrast to myocyte proliferation strategies, although the latter are generally aimed at supplanting apoptotic activity in end-stage failure. In our previous studies on analogous, post-mitotic neurons within the central nervous system, we have likewise found that cell-cycle activation and apoptosis activation characterize the disease state, but that both processes tend to be abortive and colocalize to vulnerable neurons and neurons containing pathological lesions (Ogawa et al. 2003; McShea et al. 1997; Raina et al. 2000). It appears that in post-mitotic cells, activation of the cell cycle leads to cellular dysfunction or apoptosis, rather than true, physiologically meaningful cell division. We therefore suggest a similar process in diseases affecting cardiac myocytes: that increased mechanical stress of whatever etiology leads to aberrant activation of the cell cycle, which is associated with p27 phosphorylation, cytoplasmic retention and degradation, as seen in this study, and subsequent activation of cyclin-dependent kinases. This leads in turn to cellular dysfunction, for example, by increased expression of contractile and other proteins normally reserved for fetal development (Izumo et al. 1988), and overall organ dysfunction expressed as a cardiomyopathy. Such a notion is supported by work in transgenic animals, where activation of myc, leading to cell-cycle reentry, leads to cardiac hypertrophy (Xiao et al. 2001).

Alternatively, the apoptotic cascade may be triggered, leading to programmed cell death (Narula et al. 1996; Olivetti et al. 1997). In light of the common finding of cardiac enlargement throughout the spectrum of myocardial diseases, we suspect that apoptosis is less relevant than the aberrant addition of contractile elements leading to functional impairment. In either scenario, however, the results are deleterious, and suggest that the simple cell-cycle activation may be an oversimplified concept in terms of the treatment of cardiac failure.

In summary, we demonstrate increased cytoplasmic phospho-p27 within cardiac myocytes in tissues affected by chronic ischemic heart disease

associated with cardiomyopathy and congestive failure, and an absence of evidence for cardiac myocyte proliferation *per se*. Based on this data, we suggest that aberrant cell-cycle activation occurs in response to cardiac myocyte injury, and that the addition of cardiac myocyte contractile elements as a response to injury is qualitatively deficient, thus accounting for the positive feedback loop of increasing heart size and decreasing function in disease.

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