American Journal of Pathology, Vol. 149, No. 3, September 1996

In Situ Apoptosis Assay for the Detection of Early Acute Myocardial Infarction

Ricardo H. Bardales, L. Shea Hailey, Su Su Xie, Robert F. Schaefer, and Su-Ming Hsu

From the Department of Pathology, University of Arkansas for Medical Sciences, Laboratory Services, John L. McClellan Memorial Veterans Hospital, Little Rock, Arkansas, and the Institute of Immunology, National Taiwan University College of Medicine, Taipei, Taiwan

Detection and age determination of myocardial infarction (MI) is often necessary in both clinical and pathological settings. Conventional histopathological techniques are of limited utility in the demonstration of myocardial ischemic cell death (MICD) within the first 6 hours of MI. In this study, an* in situ *apoptosis assay was evaluated for the determination of early MICD or early MI. Sections of formalin-fixed, paraffin-embedded archival tissue blocks from 80 hearts were stained for the presence of apoptotic cells by specific labeling of nuclear DNA fragmentation. Conventional hematoxylin and eosin stain showed acute MI (group A, n = 32), equivocal evidence for MICD or early infarction (group B, n = 35), or no abnormal findings (group C, n = 13). The sensitivity and specificity of the* in situ *apoptosis assay for MICD were confirmed in groups A and C patients. We showed that apoptosis of myocardial cells can occur after ischemic myocardial cell injury. Virtually all documented cases of acute MI (group A) revealed a sizeable distribution of apoptotic cells visible on gross examination of glass slides. Special attention was given to patients in group B, who were at high risk for MI and for suspected but not proved cardiac death. In this group, 34/35 cases (97%) showed focal or diffuse nuclear positivity of varying degrees for apoptosis, confirming the presence of MICD. A sizeable distribution of apoptotic cells, similar to that observed in group A, was noted in 13/35 cases (37%) of group B, suggesting acute MI in these cases. The* in situ *assay of DNA fragmentation can detect MICD while the histological diagnosis is still inconclusive. It is estimated that with this assay one can detect MICD as early as 2 to 4 hours. (Am J Pathol 1996, 149:821–829)

Normal tissue homeostasis is based on cell proliferation and cell death. Cell death, the cornerstone of balance between cell production and cell loss for a wide variety of physiological and pathological processes, is one of the most intriguing, controversial, and poorly understood topics in cell biology.[1] Apoptosis, necrosis, programmed cell death, and oncosis are terms used to describe various forms of cell death.[2]

The concept that apoptosis is a process fundamentally different from necrosis is based on its morphology, biochemistry, and incidence.[3] Apoptosis is a single-cell phenomenon characterized by compaction and margination of chromatin and by nuclear shrinkage. This is followed by nuclear fragmentation and formation of apoptotic bodies, usually with a minimal or absent inflammatory response. Necrosis, a process affecting cell groups, exhibits irregular chromatin clumping followed by swelling and disintegration of organelles and by progressive tissue damage with preservation of the overall cell configuration. The multicellular necrosis, in contrast to apoptosis, is usually accompanied by the presence of an inflammatory exudate.[4] Recently, the term oncosis has been proposed for defining cell death triggered by ischemic mechanisms.[2] Necrosis is considered the possible final outcome of both oncosis and apoptosis.[2]

Presented as a platform presentation at the 84th Annual Meeting of the United States-Canadian Academy of Pathology, Toronto, Canada, March 11–17, 1995.

Accepted for publication May 1, 1996.

Address reprint requests to Dr. Ricardo H. Bardales, Laboratory Services 113/LR, John L. McClellan Memorial Veterans Hospital, 4300 W 7th Street, Little Rock, AR 72205; or to Dr. Su-Ming Hsu, Graduate Institute of Immunology, National Taiwan University College of Medicine, 1-1 Jen-Ai Road, Taipei, Taiwan.

The apoptotic pathway may be initiated in response to various genetic, biochemical, and cellular signals. Several proteins, including *bcl*-2, p53, *myc*, and c-*fos*, regulate this complex process.[5] Internucleosomal cleavage of DNA, perhaps caused by cytoplasmic proteases signaling endonuclease activation, has become accepted as the hallmark for this form of cell death, detected as a ladder pattern by gel electrophoresis.[6] Recently, an *in situ* apoptosis (DNA fragmentation) assay (ISAA) that permits visualization of apoptosis by conventional immunohistochemistry applied to paraffin-embedded tissue sections has been described.[7,8] It is based on the specific binding of terminal deoxynucleotidyl transferase (TdT) to the 3′-OH ends of DNA. This enzyme is then used for incorporation of biotinylated deoxyuridine at DNA break sites. The ISAA has been shown to mark apoptotic cells in a distinctive pattern before morphological changes and may be sufficiently sensitive to detect the limited number of DNA strand breaks in these cells.[7,8] This technique has been applied to the study of several disorders.[9–17]

The role of apoptosis in cardiac disorders including acute myocardial infarction (MI) has been addressed previously.[14,15,18,19] Early cellular events of MI can be detected by electron microscopy but usually are not identified by light microscopy within the first 6 to 8 hours of MI.[20] Kajstura et al[15] have recently used the ISAA to study the contribution of the ischemia-induced apoptosis to the infarct size in rat myocardium. The specificity of the ISAA was confirmed by a concomitant nucleosomal ladder pattern detected on gel electrophoresis. Their results indicate that apoptosis is the major initial form of ischemic myocardial cell death occurring within the first 2 to 3 hours and that necrotic cell death follows apoptosis and contributes to the evolution of the MI.[15] We conducted the present study to evaluate the usefulness of the ISAA in the diagnosis of early MI using archival formalin-fixed, paraffin-embedded tissue sections.

Materials and Methods

Autopsy Case Selection

Files of autopsies performed on adults at the University of Arkansas for Medical Sciences and the John L. McClellan Memorial Veterans Hospital from 1988 through 1993 were reviewed. A total of 80 cases were selected on the basis of clinical history, cause of death, and initial histological interpretation of heart sections. Medical records were reviewed when necessary. Cases were classified as follows.

Group A comprised 32 patients with histological evidence of MI.

Group B included 35 patients who were at high risk for MI but had equivocal histological evidence of early MI. All of these patients had no apparent reason other than cardiac problems to explain the causes of death. Clinical diagnoses included angina of recent onset (n = 3), congestive heart failure (n = 3), ventricular tachycardia or arrhythmia (n = 4), cardiogenic shock (n = 2), and coronary bypass surgery, aortic valve surgery, and hepatectomy (one case each). The clinically suspected cause of death in 20 additional cases admitted for various reasons was a possible MI.

Group C consisted of 13 patients who had no history of heart diseases, MI, or hypertension. At autopsy, the hearts were completely normal, without evidence of infarction.

Available hematoxylin and eosin (H&E)-stained sections of myocardium from each case were reviewed. We looked for indicators of myocardial ischemia or necrosis such as nuclear changes, cytoplasmic eosinophilia, stretching and waviness of myofibers, presence of neutrophils, coagulation necrosis, contraction band necrosis, and vacuolar degeneration.

The heart weight and patency of coronary arteries were recorded if available. The normal heart weight was considered to be 250 to 300 g for women and 300 to 350 g for men. Coronary arteries were considered significantly occluded when >85% occlusion of one major vessel or >75% occlusion of more than one major vessel was present.

In Situ *Apoptosis Assay*

Sections from selected formalin-fixed, paraffin-embedded tissue blocks were placed on coated slides and stained for the ISAA as described.[7,8] Briefly, tissue sections were dewaxed and rehydrated routinely and then washed in TdT buffer (30 mmol/L Trizma base, pH 7.2, 140 mmol/L sodium cacodylate, 1 mmol/L cobalt chloride). Sections were subsequently treated with proteinase K (20 μg/ml) at room temperature (RT) for 15 minutes and washed with 1X TdT buffer for 15 minutes at RT. TdT (0.3 EU/μl) and biotinylated dUTP (20 mmol/L) in TdT buffer were added to cover the sections, followed by incubation in a humid atmosphere at 37°C. The reaction was terminated after 60 minutes by transfer of the slides to Tris-buffered saline for 5 minutes at RT. The sections were then covered with avidin-biotin-peroxidase complex, incubated for 30 minutes at RT,

Table 1. *Case Selection and Diagnoses for the Three Groups*

Group	Age (years)		Sex (male: female)	Histological diagnosis	Clinical diagnosis
	Median	Range			
A (n = 32)	70	41–86	27:5	Acute MI	Acute MI
B (n = 35)	61	28–79	28:7	Inconclusive	Inconclusive
C (n = 13)	50	33–71	13:0	WNL	Others

WNL, within normal limits.

washed with TBS, and stained with diaminobenzidine-$NiCl_2$-H_2O_2 for 5 minutes at RT.[21]

ISAA Sensitivity and Specificity and Controls

Formalin-fixed, paraffin-embedded sections of the small-intestinal mucosa and tonsil were used as staining controls. A minimum of 5% positive nuclei at the tip of the villi or germinal centers as detected by ISAA was considered adequate for staining sensitivity.[7,8] In addition, normal myocardial sections pretreated with DNAse were used as positive controls. Either TdT or biotin-dUTP was omitted in negative controls.

Previously, we have reported the results of ISAA in tissue sections of surgically resected small intestine, prostate, and tonsil kept at 4 to 6°C for 2 to 24 hours and then fixed in formalin. We observed that, regardless of the conditions of tissue preparation and fixation, the distribution of staining for apoptosis using the ISAA was similar if not identical to that seen in the surgically removed and promptly fixed specimens. Similar results were obtained in various postmortem tissues showing different degrees of autolysis. These results prove the specificity of the ISAA for detection of apoptotic cells.[22]

Results

Clinical data for each group are listed in Table 1. All 32 patients in group A had histological evidence of MI of more than 24 hours duration. Polymorphonuclear cells (PMNs) were present in 13 cases. All 35 patients in group B had inconclusive histological evidence of myocardial damage. Cardiomegaly was found in 22 cases; the heart weight was normal in 8 and unknown in 5 cases. Coronary arteries were patent in 18 cases and significantly occluded in 15; in 2, the condition was unknown. All 13 patients in group C had normal myocardial histology. The heart weight was recorded in 7 cases; all but 2 had normal weights. Coronary arteries were examined in 10 cases, and 2 had significant occlusion.

In groups A and B, myocytes with apoptotic nuclei were usually seen in groups of various sizes. On H&E stain, the nuclei appeared normal or at most to have minimal morphological changes, but apoptotic nuclear features were notoriously absent in these cells. Some nuclei may be enlarged, with chromatin condensation exhibiting a box-car-like feature. In addition, cells with diffuse cytoplasmic staining, having positive but obscured karyorrhectic nuclei, were observed. Weak cytoplasmic positivity usually was correlated with eosinophilia on H&E, and a strong reaction was correlated with necrosis. Furthermore, apoptotic cells, with either nuclear or cytoplasmic staining or both, were often visible as dark areas on gross examination of the stained sections. We define this as a geographic pattern, which was typically present in the histologically documented areas of MI, predominantly in the central zone (Table 2 and Figure 1).

In group A, all 32 cases, which had a histological diagnosis of MI, showed cytoplasmic and nuclear positivity in the corresponding infarcted areas. The center of the infarction always showed a strong cytoplasmic stain. A strong nuclear and a weaker cytoplasmic stain with stretching and waviness of myofibers were noted at the periphery of the MI (Figure 2). In some cases, the zonal distribution was not quite evident possibly as a result of improper autopsy sampling. The geographic pattern was observed in 28 cases. The remaining 4 cases (without geographic pattern) showed multiple areas of diffuse nuclear staining and scattered noncoalescent cytoplasmic staining with intermingled

Table 2. In Situ *Apoptosis Assay: Staining Results in 80 Cases*

Group	Nuclear	Cytoplasmic	Geographic
A (n = 32)	32/32 (100%)	32/32 (100%)	28/32 (88%)
B (n = 35)	34/35 (97%)	13/35 (37%)	6/35 (17%)
C (n = 13)	1/13 (8%)	0/13 (0%)	0/13 (0%)

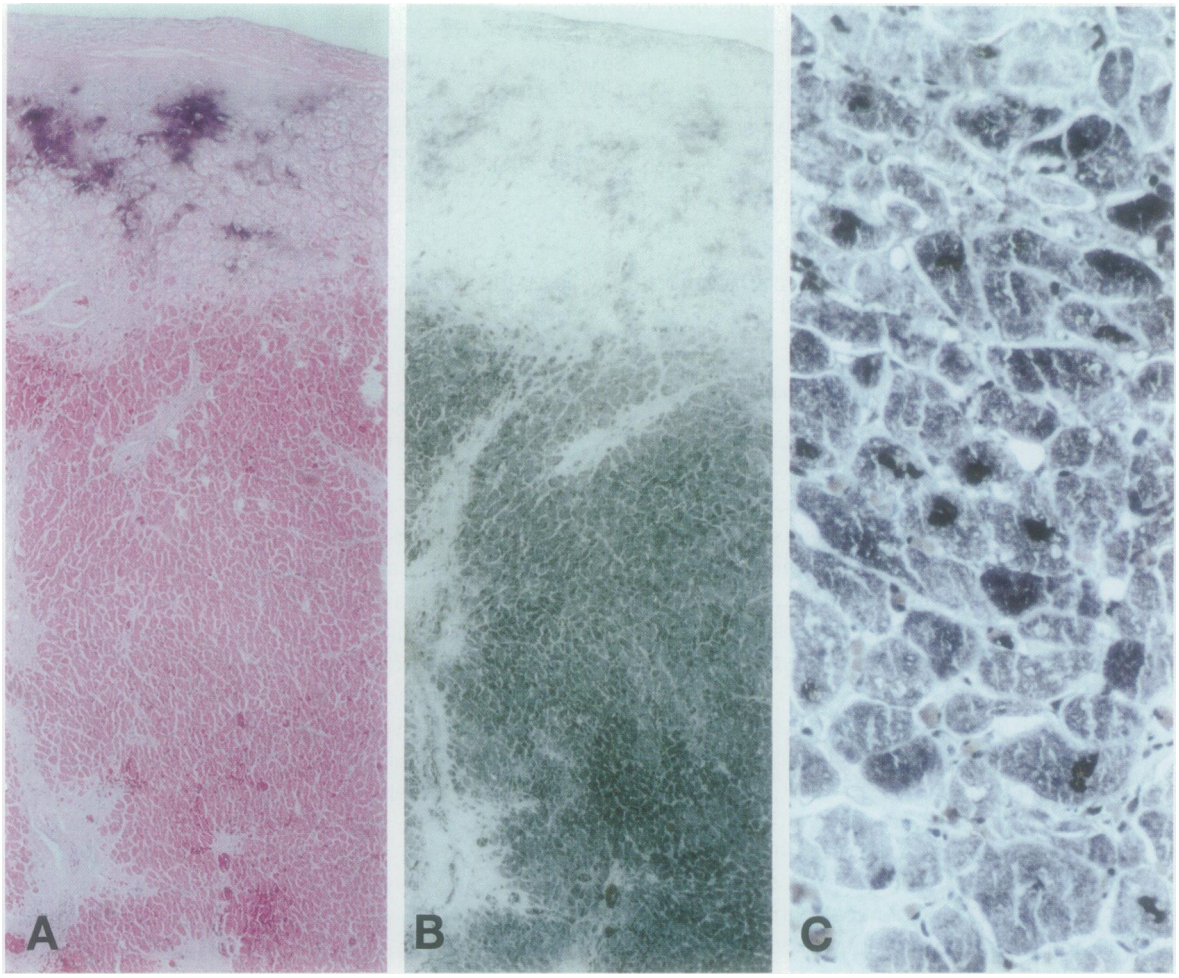

Figure 1. *Acute MI of 5 days duration was the cause of death in this 77-year-old man.* **A:** *Extensive areas of massive coagulation necrosis. H&E, magnification, ×50.* **B** *and* **C:** In situ *apoptosis assay. The infarcted area displayed a geographic pattern, which was best seen on gross examination of the glass slide. Strong cytoplasmic staining and rare positive nuclei are noted on high power view.* **B:** *Methyl green counterstain; magnification, ×50.* **C:** *Hematoxylin counterstain; magnification, ×400.*

fibrosis. Infiltrating PMNs, when present, were ISAA negative. Unaffected, histologically normal cardiomyocytes exhibited negative nuclear and cytoplasmic staining.

In group B, nuclear positivity indicating the presence of apoptosis in MICD was noted in 34 of the 35 cases. A geographic distribution of apoptotic cells, similar to that observed in group A, was seen in 6 of the 35 cases (17%), suggesting a more severe or prolonged form of myocardial ischemic injury (ie, infarction) as the cause of death. In the other 29 cases, sampling variation may have been a part of the reason for the scattered cytoplasmic staining pattern (13 cases), in most cases along with a diffuse nuclear reaction. We must emphasize that in this group heart sections were randomly sampled by the prosector because of lack of gross evidence of MI. The earliest apoptotic changes were detected in 2 patients who had died of angina of recent onset. Nuclear positive staining only and a weak geographic pattern along with nuclear positivity were found at 2 and 4 hours after the onset of angina, respectively.

In group C, except in one case, neither nuclear nor cytoplasmic positivity were detected (Figure 3), regardless of delay in postmortem examination and/or tissue fixation. Rare small foci of positive nuclei detected by ISAA were seen in a 69-year-old man who had died of massive gastrointestinal bleeding. No histological changes were detected in the corresponding H&E-stained tissue sections.

Discussion

Apoptosis is controlled by a precise intrinsic genetic program and may be induced by almost all stimuli

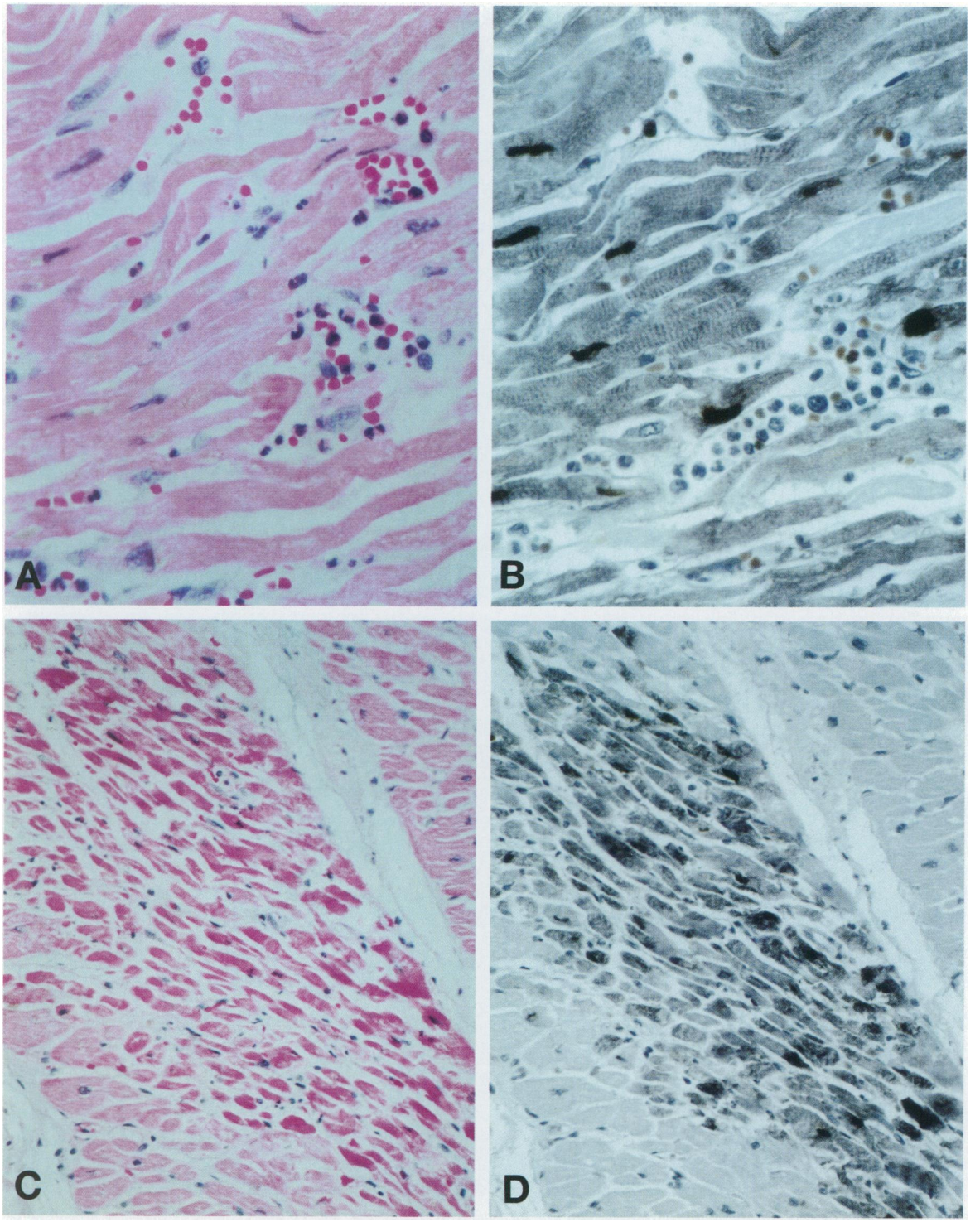

Figure 2. *Acute MI of 2 days duration.* **A** *and* **C:** *Sections of cardiac muscle revealed microscopic foci of acute MI showing minimal neutrophilic infiltrate, waviness, and eosinophilia. Histologically normal myocardial fibers are apparent around the infarcted focus.* **B** *and* **D:** In situ *apoptosis assay. Precise demarcation between infarcted and normal tissue and intense cytoplasmic staining are seen. Conspicuous striations are still preserved. Neutrophils remain negative.* **A** *and* **B:** *H&E; magnification, ×400.* **C** *and* **D:** *hematoxylin counterstain; magnification, ×200.*

that cause necrosis.[23] This concept is illustrated in Kerr's model of ischemic liver injury.[24] In his experiment, two types of cell death were described: centrilobular necrosis and peripheral apoptosis. The occurrence of both types of cell death suggests some degree of overlap, perhaps in the triggering mecha-

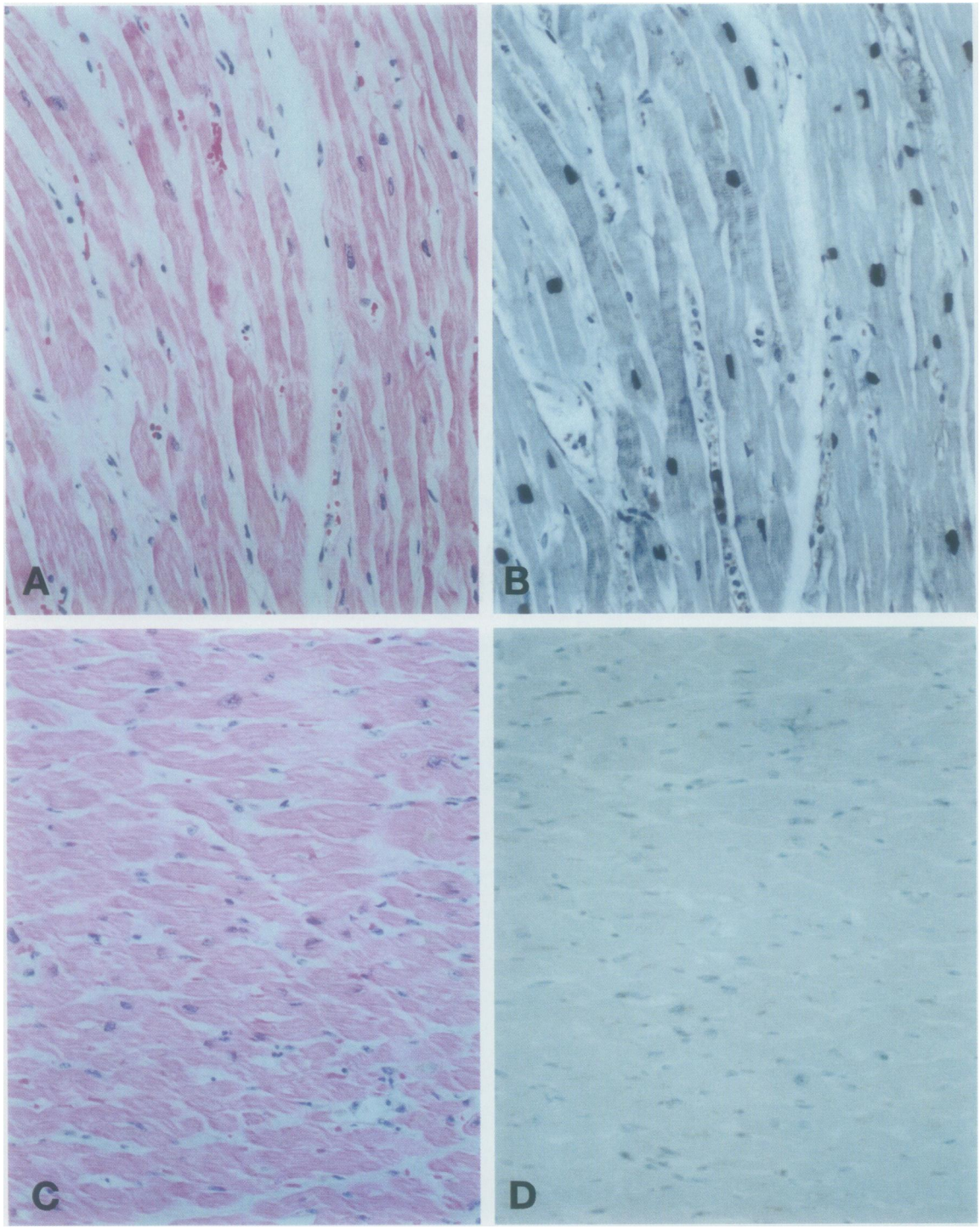

Figure 3. A: *Sections of myocardium in this 61-year-old-man who died suddenly 4 hours after onset of chest pain show equivocal myocardial cell changes. H&E; magnification, ×200.* **B:** In situ *assay for apoptosis demonstrates strong nuclear positivity and weak cytoplasmic staining, indicative of an early ischemic cell death. Hematoxylin counterstain; magnification, ×200.* **C:** *Section of normal myocardial tissue from a patient who died of carcinomatosis. The autopsy was performed 30 hours postmortem. H&E; magnification, ×200.* **D:** In situ *assay for apoptosis is negative in normal myocardial cells. Methyl green counterstain; magnification, ×200.*

nisms and cellular response, between necrosis and apoptosis.[3] The relationship between ischemia and apoptosis is also evident in other organ systems. Ischemic central nervous system injury can produce apoptotic patterns of cell death.[25–27] In rat kidney, brief periods of arterial clamping can initiate exten-

sive apoptosis, and severe and prolonged ischemia induces cellular necrosis.[28]

Apoptosis has also been detected in a variety of non-ischemic heart disorders. In the long QT syndrome, for example, the cells of the surgically excised sinus nodes show degeneration and ultrastructural changes typical of apoptosis. Both the episodic clinical onsets and the terminal event in patients with this syndrome may be due to apoptosis of the conduction system (sinus nodes) of undetermined cause.[18] In a recent review, apoptosis was implicated in many heart conditions, such as cardiomyopathy, paroxysmal arrhythmias, fibromuscular dysplasia of the small coronary arteries, arrhythmogenic right ventricular dysplasia, and some forms of myocarditis.[12,19]

There is an indication that cardiomyocyte death can be caused by apoptosis due to hypoxia.[11] The presence of apoptosis in MI has recently been supported by the observation of a nucleosomal ladder pattern on DNA electrophoresis of the infarcted myocardium,[14,15] but the triggering mechanism is not known. However, factors such as oxidative stress and production of free radicals associated with reperfusion could also trigger or exacerbate apoptosis and myocardial damage.[29] Whether reperfusion plays a key role in apoptotic myocardial cell death is subject to debate. Studies on rabbit cardiomyocytes have also shown that apoptosis is not detectable during a short period of ischemic injury (<30 minutes) but becomes evident only after reperfusion.[30] Kajstura et al[15] reported on the role of apoptosis as the earliest (detected first at 2 hours) and major form of myocardial damage. In both cases, one can attribute the lag between the onset of ischemic injury and the onset of apoptosis to the fact that the apoptotic process requires de novo mRNA, or protein (e.g., endonuclease) synthesis or enzyme activation. Not all ischemia or hypoxia causes irreversible myocardial cell injury. When the cellular damage is of sufficient extent, it reaches a point of no return, and the cells begin to institute an irreversible biochemical cascade resulting in irrevocable cell death (apoptosis).

Polymorphonuclear leukocytes are usually seen at approximately 6 hours after the onset of MI. Participation of PMNs in triggering myocardial cell death has been suggested. Youker et al[31] proposed that cardiac lymph, which contains interleukin-6 in ischemic dog hearts, makes the myocardial cells susceptible to PMN attack in cases of MI.[31] PMNs may aggravate myocyte death but probably are not involved in the initiation of apoptosis. In our ISAA, apoptotic myocardial cells can be detected long before the appearance of PMNs. Whether apoptosis is related solely to ischemia or to a combination of ischemia, reperfusion, free radicals, and other factors has not been precisely defined. Our study and those of others[14,15] strongly support the presence of this form of cell death in MI, regardless of the triggering mechanism involved after the ischemic event.

Dating and age determination in MI are often necessary. The earliest nuclear changes detected by conventional light microscopy occur at approximately 5 hours and consist of karyolysis or pyknosis, but they may be too subtle to permit a definitive diagnosis. Necrosis also begins at approximately 5 hours, a state in which the cytoplasm assumes an eosinophilic hue that is difficult to distinguish from artifactual overstaining. PMNs, the typical cells of the earliest stages of MI appear at approximately 6 hours, but they may not be present in all cases. Stretching and waviness of myofibers are not absolute as indicators of MI, because they may also be seen in a variety of other conditions. In general, conventional histological examination is unreliable for diagnosis of MI, at least within the first 6 hours.[20] Furthermore, histochemical stains and electron microscopy must be done on fresh tissue, because autolysis interferes with the results. Such methods are often not applicable to autopsy material.

The ISAA marks apoptotic cells in a distinctive pattern and has been shown to be sufficiently sensitive to detect apoptosis before morphological changes.[7,8] This method has been used for demonstration of the role of apoptotic cell death in MI.[14,15] By using the same technique to assess the age of MI, we found that the center of the infarct displays an intense cytoplasmic stain, and the periphery exhibits a strong nuclear positivity and a weak cytoplasmic stain. These results recapitulate those reported by Kerr[24] in the original description of apoptosis. As previously reported,[14,15] the nuclear positivity noted in the absence of any nuclear change detectable by light microscopy supports the fact that the DNA changes of apoptosis occur in the nucleus early in the process of MI. This is followed by karyorrhexis and by subsequent admixture of the fragmented nuclear chromatin with the cytoplasmic contents, visualized as a cytoplasmic stain that is more intense in the center than in the periphery of the infarct. In our study, these findings were easily detected in MIs older than 1 day. A geographic distribution, which correlates with involvement of a critical mass of cardiomyocytes as seen in group A, results in a life-threatening infarction.

The nuclear positivity observed in group B, similar to that present in cases of MI (group A), supports an

apoptosis-mediated myocardial ischemic injury at or near the time of death. For the cases in group B, we found that 17% of patients also exhibited a cytoplasmic staining that formed a geographic pattern. This pattern was usually seen in association with cardiomegaly and significant coronary artery disease, which are known factors associated with MI. If the heart is properly sampled or sufficient numbers of tissue blocks are examined, the presence of apoptotic cardiomyocytes will confirm the presence of antemortem ischemic injury and impending infarction.

Using the ISAA, Kajstura et al[15] detected apoptotic cell death in approximately 97% of damaged cardiac myocytes 2 hours after the occlusion of a coronary artery. Apoptosis continued to represent the major form of myocyte cell death at 4.5 to 6 hours, and myocyte necrosis (defined by disruption of cell membrane) prevailed at 1 and 2 days.[15] Thus, distinction of apoptotic cells from necrotic cells may not be necessary during the first 6 hours after the onset of MI. Later on, the presence of necrotic myocytes can be detected by light microscopy. This finding is consistent with our ISAA using archival autopsy specimens obtained from patients suspicious for MI usually less than 4 to 6 hours. We found that nuclear positive staining, the earliest change of ischemic cell death detected with this method, may be seen as early as 2 hours, but probably not before 30 minutes, as shown by a previous reperfusion study.[30] Karyorrhexis and cytoplasmic staining can be detected as early as 4 hours.

We conclude that 1) apoptosis of myocardial cells occurs early in myocardial cell injury leading to MI, 2) the *in situ* assay of DNA fragmentation can detect myocardial cell death while the histological diagnosis is still inconclusive, 3) often, in all documented cases of MI, the distribution of apoptotic cells was of sufficient size to be visible on gross examination of glass slides (ie, a geographic pattern), and 4) the observation implicating apoptosis in early myocardial ischemic cell injury may open up an early therapeutic intervention for the preservation of cardiomyocytes during an ischemic attack by preventing irreversible apoptotic events.

References

1. Farber E: Programmed cell death: necrosis *versus* apoptosis. Mod Pathol 1994, 7:605–609
2. Majno G, Joris I: Apoptosis, oncosis, and necrosis: an overview of cell death. Am J Pathol 1995, 146:3–15
3. Hockenbery D: Defining apoptosis. Am J Pathol 1995, 146:16–18
4. Wyllie AH: Cell death: a new classification separating apoptosis from necrosis. Cell Death in Biology and Pathology. Edited by ID Bowen, RA Lockshin. London, Chapman and Hall, 1981, pp 9–34
5. Stewart BW: Mechanisms of apoptosis: integration of genetic, biochemical, and cellular indicators. J Natl Cancer Inst 1994, 86:1286–1296
6. Wyllie AH, Morris RG, Smith AL, Dunlop D: Chromatin cleavage in apoptosis: association with condensed chromatin morphology and dependence on macromolecular synthesis. J Pathol 1984, 142:67–77
7. Gavrieli Y, Sherman Y, Ben-Sasson SA: Identification of programmed cell death *in situ* via specific labeling of nuclear DNA fragmentation. J Cell Biol 1992, 119:493–501
8. Wijsman JH, Jonker RR, Keijzer R, Van de Velde CJH, Cornelisse CJ, Van Dierendonck JH: A new method to detect apoptosis in paraffin sections: *in situ* end-labeling of fragmented DNA. J Histochem Cytochem 1993, 41:7–12
9. Ansari R, Coates PJ, Greenstein BD, Hall PA: *In situ* end-labeling detects DNA strand breaks in apoptosis and other physiological and pathological states. J Pathol 1993, 170:1–8
10. Fukasawa Y, Ishikura H, Takada A, Yokoyama S, Imamura M, Yoshiki T, Sato H: Massive apoptosis in infantile myofibromatosis: a putative mechanism of tumor regression. Am J Pathol 1994, 144:480–485
11. Tanaka M, Ito H, Adachi S, Akimoto H, Nishikawa T, Kasajima T, Marumo F, Hiroe M: Hypoxia induces apoptosis with enhanced expression of Fas antigen messenger RNA in cultured neonatal rat cardiomyocytes. Cir Res 1994, 75:426–433
12. Kawano H, Okada R, Kawano Y, Sueyoshi N, Shirai T: Apoptosis in acute and chronic myocarditis. Jpn Heart J 1994, 35:745–750
13. Kasagi N, Gomyo Y, Shirai H, Tsujitani S, Ito H: Apoptotic cell death in human gastric carcinoma: analysis by terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling. Jpn J Cancer Res 1994, 85:939–945
14. Itoh G, Tamura J, Suzuki M, Suzuki Y, Ikeda H, Koike M, Nomura M, Jie T, Ito K: DNA fragmentation of human infarcted myocardial cells demonstrated by the nick end-labeling method and DNA agarose gel electrophoresis. Am J Pathol 1995, 146:1325–1331
15. Kajstura J, Cheng W, Reiss K, Clark WA, Sonnenblick EH, Krajewski S, Reed JC, Olivetti G, Anversa P: Apoptotic and necrotic myocyte cell deaths are independent contributing variables of infarct size in rats. Lab Invest 1996, 74:86–107
16. Desmouliere A, Redard M, Darby I, Gabbiani G: Apoptosis mediates the decrease in cellularity during the transition between granulation tissue and scar. Am J Pathol 1995, 146:56–66
17. Kressel M, Groscurth P: Distinction of apoptotic and necrotic cell death by *in situ* labelling of fragmented DNA. Cell Tissue Res 1994, 278:549–556
18. James TN, Terasaki F, Pavlovich, Vikhert AM: Apopto-

sis and pleomorphic micromitochondriosis in the sinus nodes surgically excised from five patients with the long QT syndrome. J Lab Clin Med 1993, 122:309–323
19. James TN: Normal and abnormal consequences of apoptosis in the human heart: from postnatal morphogenesis to paroxysmal arrhythmias. Circulation 1994, 90:556–573
20. Lodge-Patch I: The aging of cardiac infarcts and its influence on cardiac rupture. Br Heart J 1951, 13: 37–42
21. Hsu SM, Raine L, Fanger H: Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: a comparison of ABC and unlabeled antibody (PAP) procedure. J Histochem Cytochem 1981, 29:577–580
22. Bardales RH, Morrow M, Moody MJ, Xie SS, Hsu SM: *In situ* DNA fragmentation assay to detect apoptosis in paraffin-embedded tissue sections: technical considerations. Mod Pathol 1995, 8:162a
23. Sen S: Programmed cell death: concept, mechanism, and control. Biol Rev Cambridge Phil Soc 1992, 67: 287–319
24. Kerr JFR: Shrinkage necrosis: a distinct mode of cellular death. J Pathol 1971, 105:13–20
25. MacManus JP, Hill IE, Huang ZG, Rasquinha I, Hue D, Buchan AM: DNA damage consistent with apoptosis in transient focal ischemic neocortex. Neuroreport 1994, 5:493–496
26. MacManus JP, Buchan AM, Hill IE, Rasquinha I, Preston E: Global ischemia can cause DNA fragmentation indicative of apoptosis in rat brain. Neurosci Lett 1993, 164:89–92
27. Linnik MD, Zobrist RH, Hartfield MD: Evidence supporting a role for programmed cell death in focal cerebral ischemia in rats. Stroke 1993, 24:2002–2008
28. Schumer M, Colombel MC, Sawczuk IS, Gobe G, Connor J, O'Toole KM, Olsson CA, Wise GJ, Buttyan R: Morphologic, biochemical, and molecular evidence of apoptosis during the reperfusion phase after brief periods of renal ischemia. Am J Pathol 1992, 140:831–838
29. Engler R, Gilpin E: Can superoxide dismutase alter myocardial infarct size? Circulation 1989, 79:1137–1142
30. Gottlieb RA, Burleson KO, Kloner RA, Babior BM, Engler RL: Reperfusion injury induces apoptosis in rabbit cardiomyocytes. J Clin Invest 1994, 94:1621–1628
31. Youker K, Smith CW, Anderson DC, Miller D, Michael LH, Rossen RD, Entman ML: Neutrophil adherence to isolated adult cardiac myocytes: induction by cardiac lymph collected during ischemia and reperfusion. J Clin Invest 1992, 89:602–609