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Evans Blue Staining of Cardiomyocytes Induced by Myocardial Contrast Echocardiography in Rats: Evidence for Necrosis Instead of Apoptosis

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Abstract

High Mechanical Index (MI) echocardiography with contrast agent has been shown to induce Evans blue staining of cardiomyocytes, seen one day after exposure, in addition to contraction band necrosis, seen immediately after exposure. This research examined the roles of necrosis vs. apoptosis in these bioeffects. Myocardial contrast echocardiography at high MI with 1:4 ECG triggering was performed in anesthetized rats at 1.5 MHz. Histologically observable cell injury was accumulated by infusing a high dose of 50 μ l/kg Definity® *via* tail vein for 5 min at the start of 10 min of scanning. Evans blue dye or propidium iodide was injected as an indicator of cardiomyocyte plasma membrane integrity. Histological sections were stained using the TUNEL method for labeling nuclei with DNA degradation (e.g. apoptosis). Evans blue fluorescent cells were counted on frozen sections or on hematoxylin-stained and TUNEL labeled paraffin sections. In addition, transmission electron microscopy was used to assess potential apoptotic nuclei. Hypercontraction and propidium iodide staining were observed immediately after imaging-exposure. Although TUNEL positive cells were evident after 4 h, these also had indications of contraction band necrosis and features of apoptosis were not confirmed by electron microscopy. Inflammatory cell infiltration was evident after 24 h. A second, more subtle injury was recognized by Evans blue staining, with minimal inflammatory cell infiltration at the morphologically intact stained cells after 24 h. Apoptosis was not detected by the TUNEL method in the cardiomyocytes stained with Evans blue at 24 h. However, Evans blue stained cell numbers declined after 48 h, with continued inflammatory cell infiltration. The initial insult for Evans blue stained cardiomyocytes apparently induced partial permeability of the plasma membrane, which led to gradual degeneration (but not apoptosis) and necrosis after 24–48 h.

Keywords

cardiomyocyte death; apoptosis; necrosis; ultrasound contrast media; ultrasonic cavitation; biological effects of ultrasound

INTRODUCTION

The interaction between ultrasound pulses and gas bodies is an important mechanism for nonthermal bioeffects of ultrasound (NCRP, 2002). Myocardial contrast echocardiography

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(MCE) using diagnostic ultrasound scanners at high Mechanical Index (MI) has been associated with capillary rupture in the myocardium resulting in petechiae-like erythrocyte extravasation sites in animal models (Ay et al. 2001; Li et al. 2004). In addition, histological evidence of cardiomyocyte contraction band necrosis immediately after exposure has been shown for diagnostic ultrasound with commercial contrast agent in rats (Miller et al. 2005a). This injury and cell death led to inflammatory cell infiltration within identifiable microlesions the next day. However, cardiomyocyte injury was also found to be detectable by staining with Evans blue dye, which was detected the day after exposure (Miller et al. 2005b). The incidence of the Evans blue staining depended on a complex set of parameters including ultrasound MI, contrast agent dose, intermittent image timing and dose infusion timing (Miller et al. 2005c). The Evans blue stained cells evident after 24 h seemed to be a separate type of cardiomyocyte injury, which appeared different from the rapid necrosis with the development of inflammatory cell microlesions within 24 h. The Evans blue stained cells appeared morphologically intact and free of inflammatory cell infiltration after 24 h: their origin is presently uncertain.

In general, cell death occurs *via* two different processes: necrosis and apoptosis (or programmed cell death) (Kumar et al. 2005). Necrosis can be caused by various injurious stimuli including mechanical disruption and ischemia, and is characterized by initial cytoplasmic degeneration and plasma membrane failure with increased permeability, which precedes nuclear degeneration with DNA breakdown. This type of cell death usually induces a significant inflammatory response (numerous inflammatory cells clearing out the dead cell debris) particularly if several cells in the same region are affected. By contrast, apoptosis is initiated by generally different injurious stimuli (e.g. radiation damage, or cytotoxic T cell killing) and is characterized by early intracellular activation of caspase and DNA enzyme activity leading to DNA degeneration and chromatin condensation, before cytoplasmic and plasma membrane degeneration. This type of cell death typically produces a minimal inflammatory response, usually in the form of individual macrophages engulfing apoptotic bodies (budded-off portions of cytoplasm). A given injury may produce both forms of cell death.

Apoptosis has been identified in various cardiac disease states, for example, in ischemia and reperfusion injury (Krijnen et al. 2002; Eefting et al. 2004; Matsushita et al. 2005; Liang et al. 2006) or doxorubicin toxicity (Ueno et al. 2006). Evans blue staining of myocytes has been associated with apoptosis in muscular dystrophy (Matsuda, 1995). Furthermore, ultrasound exposure has been shown to induce apoptosis. *In vitro* exposure of cells induced apoptosis in various cell lines (Takeuchi et al. 2006; Kinoshita et al. 2007), particularly with added contrast agents (Feril et al. 2003). *In vivo*, ultrasound exposure of rabbit brain with contrast agent gas bodies in the circulation has been shown to induce apoptosis in brain cells (Vykhodtseva et al. 2006). Apoptosis is a well known pathway of cell death of cardiomyocytes, and contrast-aided ultrasound has been shown to induce apoptosis in several cell types under different conditions. Apoptosis might therefore play an important role in *in vivo* cardiomyocyte injury by myocardial contrast echocardiography.

The induction of apoptosis in cardiomyocytes by MCE would be of interest, because this apparently has not been identified previously. The purpose of this present study was to follow, *via* histology, the progression of high dose MCE-induced cardiomyocyte injury using vital staining methods. An improved understanding of the Evans blue staining of injured cardiomyocytes was sought by evaluation of samples at different time points. The vital stain propidium iodide was tried as a means to identify necrotic cells immediately after exposure. The possible operation of apoptosis in this progression was also investigated using the terminal dUTP nick-end labeling (TUNEL) assay for apoptosis after 4 h, 1d and 2 d, and transmission electron microscopy was also employed to clarify light microscopy observations. Finally, the Evans blue staining and development of inflammatory cell microlesions was followed for 2

days. The results indicate a complex evolution of the initial injury culminating in necrosis and cell removal.

METHODS

Animal preparation

All *in vivo* animal procedures were conducted with the approval and guidance of the University Committee on Use and Care of Animals. The experimental preparation has been described previously (Miller et al. 2005a). Briefly, CD hairless rats (Charles River) were anesthetized by intraperitoneal injection of a mixture of ketamine (87 mg ml^{-1}) and xylazine (13 ml kg^{-1}). A 24 gauge cannula was inserted into a tail vein for injections. Water-proof electrodes (LL911, Lead-Lock, Inc., Sandpoint ID) were applied to three legs for ECG acquisition by the ultrasound machine. Evans blue dye in saline at a dose of 100 mg/kg was injected as a vital stain for cardiomyocytes (an indicator of plasma membrane permeability). Alternatively, propidium iodide was injected at 10 mg/kg immediately after exposure as a vital dye. The rats then were mounted on a holding board and placed in a 37°C degassed water bath for ultrasound scanning.

Ultrasound

A GE Vingmed System V (General Electric Co., Cincinnati OH) with a cardiac phased array probe (FPA2.5) was employed as the ultrasound source. The probe was clamped in the water bath to image the rat heart located 4–5 cm from the transducer face. An initial image was obtained at 3.6 MHz for aiming the probe, and then the frequency was switched to 1.5 MHz with a 60 Hz frame rate. The peak rarefactional pressure amplitude (PRPA) was 2.3 MPa with a pulse duration of $1.45 \mu\text{s}$, measured as described previously (Miller et al. 2005a). The *in situ* PRPA was estimated to be 2.0 MPa after adjustment for the minimum estimated attenuation of 12% (-1.2 dB) through the rat chest wall, which corresponds to an estimated MI of 1.6. For comparison, an $\text{MI} > 0.8$ may be considered “high” MI for contrast aided diagnostic ultrasound (Duck, 2007), although it should be noted that whether or not the MI represents a good predictor of bioeffects is uncertain. MCE intermittent images were triggered from the ECG signal each fourth heart beat at end systole.

Ultrasound Contrast Agent

Definity® (Bristol-Myers Squibb Medical Imaging, Inc., N. Billerica, MA) was prepared fresh each day. Definity® is representative of presently available ultrasound contrast agents, and effects with other agents might be expected to be similar when based on gas-body dose (Miller et al. 2004). For infusion, the agent first was diluted 50:1 in sterile saline in a 5 ml syringe, which was then mounted in a syringe pump (model 11plus, Harvard Apparatus, Holliston MA). A 30 cm extension tube was pre-loaded with diluted contrast agent and was connected between the syringe and the tail vein cannula. The volume infusion rate was set to $500 \mu\text{l/kg/min}$ ($10 \mu\text{l/kg/min}$ of the agent) and timed for 5 min (giving a total agent dose of $50 \mu\text{l/kg}$). For MCE ultrasound exposure, the scanning duration was 10 min with 5 min of infusion followed by an additional 5 min of triggered scanning. For sham exposure, the two conditions of ultrasound alone and contrast agent alone were performed separately (i. e., echocardiography for 10 min, followed by injection of the contrast agent with the ultrasound scanhead aimed away from the rat). These high-dose conditions were employed to allow clear identification of the scanned tissue for histology sampling and a substantial accumulation of effected cells for definitive evaluation.

Measured Endpoints

H&E staining was used for general evaluation of tissue samples, with serial sections left unstained for different tests (described below). Hearts were removed, cleared of most blood with heparin-saline, and cut down to the apparent scan plane, which was normally marked by a red (capillary hemorrhage) or blue (Evan blue leakage) band across the heart. The sample was then fixed in neutral buffered formalin and slides were made at the Research Histology and Immunoperoxidase Laboratory of the University of Michigan Comprehensive Cancer Center Tissue Core. Slides were typically made each 0.5 mm cutting into the sample, with several sequential slides made at each depth. After preliminary examination, the depth with the most evident tissue injury (presumably from the center location exposed to the PRPA) was selected for detailed evaluation. H&E histology was useful for detecting contraction band necrosis (also called coagulative myocytolysis (Baroldi, (2001)) of cardiomyocytes immediately after exposure, and for identifying areas with inflammatory cell infiltration after 1 d or 2 d. In addition, microlesions defined by clusters of infiltrating inflammatory cells were identified on H&E or hematoxylin-only slides, as described previously (Miller et al. 2005a). The microlesion areas were quantified on photomicrographs made using a 40 x objective in the effected region of the section (i. e., the anterior left ventricular wall) by manually outlining the affected regions and calculating the area involved with image analysis software (SigmaScan 5.0, SPSS Inc. Chicago IL). In this study, the area occupied by microlesions was compared to the area of Evans blue fluorescent cells in photographs of slides stained with hematoxylin only.

Propidium iodide enters cells with compromised plasma membranes and stains nucleic acids (e.g. nuclear DNA) to yield red fluorescence. This allows for assessment of cardiomyocyte death (Wolff et al. 2000), which had previously been shown to be useful for staining of cardiomyocytes after MCE (Miller et al. 2006). Propidium iodide staining was evaluated on slides made as described above for H&E staining, but scored on an adjacent serial section without the use of any stain. Unfortunately, this method was difficult to quantify *in vivo* due to confounding factors of non-specific red fluorescence in tissue samples, and the cells with stained nuclei could not be counted or scored unambiguously over larger areas.

Evans blue was administered one day before sacrifice (including at the time of exposure) and then observed in samples taken at least 4 h after exposure. This dye, which becomes intensely red fluorescent when conjugated to albumin in blood plasma, could not be used at earlier times after injection, because sufficient fluorescent dye remained in the circulation to confound the evaluation of cardiomyocyte staining. The dye/albumin complexes are excluded from cells with intact plasma membranes, thus providing a dye-exclusion viability test. The evaluation of Evans blue fluorescent staining was most clearly evident in frozen sections of heart samples, which were made each 0.5 mm into the sample as described previously (Miller et al. 2005b, 2005c). However, the Evans blue fluorescence was evident, though less bright, in formalin fixed paraffin sections, which could be either unstained, or stained with hematoxylin only.

De-waxed slides from adjacent serial sections were stained by the TUNEL assay (ApopTag® kit, Serologicals Corporation, Norcross, GA.), using the peroxidase labeling method with a hematoxylin counter-stain. Positive control specimens were processed with each batch of heart samples to assess the performance of the assay. Cardiomyocyte nuclei could be qualitatively distinguished from other cell nuclei (endothelial cells, blood cells) in the sections, which allowed the scoring of the fraction of TUNEL stained cardiomyocyte nuclei. For samples with Evans blue, it was possible to detect Evans blue (fluorescent cell) staining on the Apoptag® slides, because these were counter-stained with hematoxylin only.

Due to ambiguity in the interpretation of the TUNEL assay, which can lead to false positive indications of apoptosis (Ohno et al. 1998; Saraste et al. 2000; Garrity et al. 2003), transmission electron microscopy (TEM) was also employed to clarify light microscopy observations of

cardiomyocyte nuclei and cytoplasm using morphological criteria. The heart samples were cut down to millimeter sized portions of the injured regions (or similar positions in the hearts for control and sham samples) and fixed in glutaraldehyde fixative, with post fixation in osmium tetroxide. Processing and transmission electron microscopy were performed at the Microscopy and Image Analysis Laboratory of the University of Michigan Department of Cell and Developmental Biology. Epon imbedded samples were cut for 70 nm sections, which were stained with uranyl acetate and lead citrate for final viewing. The electron microscopy was particularly useful for assessing the contraction band necrosis and the possible chromatin condensation at the margin of the nucleus, which occurs for apoptosis (see, for example, Matsushita et al. 2005).

The study was conducted in different groups using a total of 59 rats. Of the total, 10 were used for propidium iodide staining, 8 for TEM, 11 for frozen sections with Evans blue, 9 for TUNNEL staining (without Evans blue) and 21 for H&E, Evans blue and TUNEL staining. Numerical results are presented as the mean plus/minus one standard deviation, or plotted as the mean with standard error bars, for 3–6 measurements in different rats. For statistical analysis, Student's t-tests or the Mann-Whitney rank sum test, as appropriate, were used to compare means of the measured parameters, with statistical significance assumed at $P < 0.05$.

RESULTS

Propidium iodide was used for evaluating cell death in heart samples taken at 15 min post exposure. An example of a red fluorescent image is shown in Fig. 1a with four stained nuclei, which were likely cardiomyocyte nuclei. A serial section stained with hematoxylin showed that cells corresponding to the vicinity of the propidium iodide stained nuclei in the adjacent slide appear to be injured, having signs of contraction band necrosis in Fig. 1b. Unfortunately, there was a gradation of red fluorescence on the sections, which did not allow for quantitative scoring.

The possible development of apoptosis in cardiomyocytes injured by MCE was studied using 3 rats each as sham, 4 h post exposure and 24 h post exposure (without Evans blue staining). An example of the result for a 4 hr test is shown in Fig. 2. The cells with nuclei lightly stained by the ApopTag® procedure also displayed hypercontraction, which was indicative of contraction band necrosis. Results for the counts of TUNEL positive nuclei, which appeared to be cardiomyocyte nuclei (from their relatively large size and position within the cells), are plotted in Fig. 3. At 4 h post MCE, there was a significant increase of TUNEL positive cardiomyocyte nuclei ($P < 0.01$) with 235 ± 55 stained nuclei counted over the entire sections. At 24 h, there were few TUNEL positive cardiomyocyte nuclei (no significant difference from shams) such as those seen in the 4 h samples.

The TUNEL positive cardiomyocyte nuclei noted after 4 h also displayed hypercontraction, which was indicative of contraction band necrosis, and the chromatin did not appear to be condensed. These features were not characteristic of the morphology of classic apoptosis. Transmission electron microscopy was performed on sham, 15 min, 4 hr and 24 h post exposure samples (2 rats each) in order to determine the state of chromatin condensation in cells with contraction band necrosis. Sham samples contained cardiomyocytes with the normal banded myofibrils, mitochondria and nuclei, as shown in Fig. 4a. There were also numerous capillaries, which were largely free of blood cells due to saline perfusion of the heart samples. In the 15 min and 4 h post exposure samples, there were instances of cardiomyocytes with a normal appearance, but with extravasated blood cells evident in the interstitium (Fig. 4b). In addition, there were cardiomyocytes with hypercontracted bands, as shown in Fig. 4c, which was the electron microscopy representation of the contraction band necrosis shown in Fig. 2. The nuclei in these cells did not have the characteristic condensed chromatin of apoptotic cell nuclei,

confirming the optical observations of non-condensed chromatin in these cells. In samples taken one day after exposure, inflammatory cells, probably neutrophils, could be seen in regions adjacent to cells which appeared to be relatively normal, although some had contracted bands, as in Fig. 4d. The closely packed inflammatory cells corresponded to the microlesions seen in optical microscopy. The Evans blue stained cardiomyocytes could not be identified in the TEM, but some intact cardiomyocytes had contracted (but not hypercontracted) bands (Fig. 4d), which may correspond to the Evans blue stained cardiomyocytes.

Staining of cardiomyocytes with Evans blue appeared to show a qualitatively different type of injury, which lacked infiltration of inflammatory cells within 24 h. Rat hearts were evaluated at 4 h and 24 h post-exposure and sham by Evans blue staining of cardiomyocytes using frozen sections. Results are listed in Table I. Cells with contraction band necrosis could not be discerned clearly on the frozen sections. The regions with inflammatory cell microlesions in the 24 h samples often showed some Evans blue fluorescence, as noted previously (Miller et al. 2005c). There was no significant difference between the cell counts for the two time points.

In order to evaluate possible apoptosis of cardiomyocytes stained with Evans blue, groups of rats were sham exposed or exposed with 1 d and 2 d post exposure delay to sacrifice. Figures 5a and 5b show an area with Evans blue fluorescent cardiomyocytes on an ApopTag® slide for a 1 day post exposure sample in transmitted and fluorescence illumination, respectively. The Evans blue stained cardiomyocytes in such slides did not have clearly TUNEL positive nuclei (although some TUNEL positive nuclei were seen in microlesion areas, as noted below). The cytoplasm of the Evans blue stained cells appeared to have a different texture from the normal cells, possibly due to contracted bands, but they typically did not have clear indications of contraction band necrosis. The Evans blue fluorescence was evaluated in the ApopTag® processed slides. The sham exposed slides were negative for Evans blue staining. The 1 d and 2 d slides were scored for Evans blue fluorescent cell counts, and the results are listed in Table I. The Evans blue fluorescent cell count for 1 d post exposure samples on these slides was not significantly different from the counts from the result from frozen sections ($P=0.11$). However, the counts of Evans blue stained cells for the 2 day post-exposure samples were significantly less than for the 1 day ApopTag® slides.

Microlesions (clusters of inflammatory cells) were not evident in the 4 hr post exposure samples, but were well-defined after 1d. The areas identified as microlesions averaged 5.3 ± 2.2 % and Evans blue fluorescent cell areas averaged 11.9 ± 7.5 in the effected areas of four sections after 24 h. As noted above, very few TUNEL positive cardiomyocytes were seen after 1d or 2 d, but TUNEL positive nuclei could be seen in other cells. Figure 6a shows several TUNEL positive nuclei within a microlesion area after 2 d. After 2d, the microlesion areas appeared more clear of eosin-stained material than after 1 d and also appeared to have significant ongoing infiltration of inflammatory cells, as shown in Fig. 6b. The Evans blue stained cardiomyocytes were typically difficult to identify as distinctly separate from the regions of inflammatory cell infiltration after 2d. This indicates removal of the dead cardiomyocytes by phagocytic cells in the normal inflammation and healing process.

DISCUSSION AND CONCLUSIONS

Myocardial contrast echocardiography at high MI can induce capillary hemorrhage and cardiomyocyte injury by the interaction of the ultrasound with contrast agent gas bodies (a form of ultrasonic cavitation). Previously, capillary hemorrhage and cells with contraction band necrosis were shown to lead to inflammatory cell infiltration and microlesions after one day (Miller et al. 2005a). However, additional research showed that many more injured cardiomyocytes could be identified after one day by the vital stain Evans blue (Miller et al. 2005b), which labels the entire cells with bright red fluorescence. The existence of the

additional killed cells was puzzling and conceivably could indicate the operation of the two cell death pathways (i. e., apoptosis, as well as necrosis). Ultrasonic cavitation has most often been associated with cell rapid necrosis (i. e., immediate mechanical cell membrane disruption at the time of exposure); however, apoptosis recently has been reported *in vitro* (Feril et al. 2003) and *in vivo* (Vykhodtseva et al. 2006) for ultrasound aided by contrast agents. The purpose of this study was to better characterize the Evans blue stained cardiomyocytes with regard to the type of cell death seen: apoptosis vs. necrosis.

At some sites, the interaction of ultrasound pulses with a gas body in the microvasculature caused rupture of the capillary and permeabilization or disruption of the cell membrane of a nearby cardiomyocyte. Mechanical permeabilization is known to cause hypercontraction due to an influx of calcium (Karpati and Carpenter, 1982). The mechanical disruption and hypercontraction allows staining by propidium iodide, a vital stain which labels nuclei in necrotic cells with red fluorescence, as shown in Fig. 1. Counting of the killed cells detected by propidium iodide was not possible over the sections due to variation in the fluorescence and the presence of red spots with uncertain origin. However, this form of cell death represents the rapid cell necrosis expected from violent cavitation activity.

TUNEL positive cardiomyocyte nuclei were seen after 4 h (Fig. 2). These cells also had indications of contraction band necrosis but not of chromatin condensation (Fig. 4). The numbers of TUNEL positive cardiomyocyte nuclei were greatly reduced after 24 h (Fig. 3). The TUNEL assay has some uncertainty with regard to identification of true apoptosis, as noted in Methods. Other features of the TUNEL positive cardiomyocytes were most consistent with necrosis, showing cytoplasmic degeneration leading to inflammatory cell infiltration. However, an early initiation of apoptotic processes, which were rapidly overtaken by necrosis, could also explain the observations. During experimental myocardial infarction in rats, apoptosis represented a major form of cardiomyocyte death at 4.5 h, with necrosis peaking at one day after coronary occlusion (Kajstura et al. 1996). In the case of MCE induced cardiomyocyte injury reported here, the mechanical injury occurs precisely at the time of exposure, which may speed the progression of cell death processes.

Evans blue staining was evident in as many cells 4 h post exposure as at 24 h post exposure (Table 1), which indicates the loss of the ability to exclude the dye occurs soon after the injury by MCE. The nuclei of Evans blue stained cardiomyocytes were not stained clearly by the ApopTag® procedure after 24 h, when the number of TUNEL positive cardiomyocytes was reduced (Fig. 3), nor was apoptosis indicated in cardiomyocytes by morphologic signs characteristic of apoptotic nuclei (Fig. 4). As the death of the Evans blue stained cells progressed their number was reduced after 2 d (Table 1), apparently by inflammatory cell phagocytosis. It seems highly likely that apoptotic cardiomyocytes would have been detected if they were present at 24 h, because the typical apoptosis of infiltrating cells, which has been shown to be involved in removal of macrophages from sites of cardiac injury (Takemura et al. 1998; Zhao et al. 2004), was readily detected among infiltrating inflammatory cells in the ApopTag® slides of 2 d post-exposure samples (Fig. 6a).

The Evans blue stained cardiomyocytes, which were intact cells with minimal inflammatory cell infiltration after 24 hr, represented a substantial component of the overall cardiomyocyte death. However, these cells were not identifiable as apoptotic. The Evans blue stained cardiomyocytes may have received a lethal mechanical injury at the time of exposure, although briefly delayed injury may have occurred from communication via gap junctions (Garcia-Dorado et al. 2004), or by the activity of the infiltrating inflammatory cells (Vinten-Johansen, 2004), which are processes implicated in cardiomyocyte injury. The Evans blue staining indicated permeabilization of the cardiomyocyte plasma membrane, which apparently led to

gradual degeneration, necrosis and removal by the normal inflammatory and phagocytic healing process after about 1–2 d.

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Figure 1.

(a) A fluorescence image of a section of a rat heart sampled within 15 min of MCE with staining by propidium iodide (arrows), which stains the nuclei of necrotic cells fluorescent red. (b) An image of the same area from a serial section slide stained with only hematoxylin showing cardiomyocytes with signs of injury (arrows) at points corresponding to the brightest propidium iodide stained nuclei in (a). Scale bars: 100 μ m.

Figure 2.

A photomicrograph of a section of a heart sample 4 hr after MCE and stained using the ApopTag® system, which shows TUNEL positive cardiomyocyte nuclei (A). The stained cells also show characteristics of contraction band necrosis (extensive disruption of the sarcomeric architecture). Normal cardiomyocyte nuclei (N) appear blue. Scale bar: 50 μ m.

Figure 3. Counts of TUNEL positive cardiomyocyte nuclei, as shown in Fig. 2. These appeared after 4 h ($P < 0.01$ relative to shams) and greatly declined after 24 hr (no significant difference from shams).

Figure 4.

(a) Electron photomicrograph of a control sample showing a capillary (C), nuclei (N), and normal banding of myofibrils with dark mitochondria between them. (b) An electron photomicrograph from a sample obtained 15 min after MCE showing several erythrocytes (three with arrows) in the interstitium outside of a capillary. (c) An electron photomicrograph from a sample obtained 15 min after MCE showing a cardiomyocyte with hypercontracted myofibrils and swollen mitochondria, but an intact nucleus without chromatin condensation (arrow). (d) An electron photomicrograph from a sample obtained 24 h after MCE showing a part of a microlesion with inflammatory cells on the left, and a contracted but relatively normal cell on the right. Scale bars: 5 μ m.

Figure 5.

(a) A photomicrograph of a section of a heart sampled after 24 h and stained using the ApopTag® method: only one nucleus has brown staining (arrow) which does not appear to reside in a cardiomyocyte. (b) A fluorescence image of the same region as in (a) showing many Evans blue stained cardiomyocytes (bright red fluorescence), none of which appear to have TUNEL positive nuclei in (a). Scale bars: 100 μ m.

Figure 6.

(a) A photomicrograph of a section stained by the ApoptTag® process from a sample taken 48 h after MCE with a microlesion defined by the inflammatory cell infiltration (numerous clustered dark blue nuclei) and with several nuclei of the infiltrated cells stained TUNEL positive (dark brown) for apoptosis (arrows). There were no Evans blue stained cells in this region. Scale bar: 50 μ m. (b) An H&E stained section taken 48 h after MCE showing microlesions with loss of eosin-stained cardiomyocytes and numerous infiltrating cells (narrow rows of dark blue-stained nuclei). Scale bar: 50 μ m.

Table I

Results for counts of Evans blue fluorescent stained cells at different times post-exposure, and using different slide processing methods (see text).

Post exposure time	n	mean	standard deviation	P value
Frozen section slides:				
4 hour	5	678	229	0.86
24 hour	6	730	600	
ApopTag® slides:				
1 day	4	1361	409	<0.01
2 day	3	280	121	