Reversible ischemia caused by brief coronary artery occlusions is not associated with tissue necrosis. Irreversible injury begins when the occlusion duration exceeds 15 minutes and the wave front of injury progresses from endocardium to epicardium.1 Studies in animals and humans have demonstrated that the mechanism of irreversible injury in infarction involves necrosis as well as apoptosis.2 Evidence is available indicating that these are independent forms of cell death in this setting,3 and interventions to reduce irreversible injury, such as preconditioning, appear to preferentially attenuate apoptosis-mediated cell death.4

Subendocardial ischemia caused by increased demand or a partial coronary occlusion can be tolerated for prolonged periods of time without the development of irreversible injury or myocyte necrosis. It is not clear, however, whether reversible ischemia can be associated with a chronic increase in myocyte apoptosis. Biopsies from patients with hibernating myocardium subjected to chronic intermittent ischemia have demonstrated rare apoptotic myocytes by electron microscopy without necrosis, but the frequency was too low to quantify.5 Other studies however, have failed to identify apoptosis in biopsies from humans with hibernating myocardium.6 Because of substantial variations in the amount of replacement fibrosis among these studies (11% to 58%) and the inability to quantify the effects of apoptosis on myocyte loss via estimates of myocyte nuclear density, it remains unknown whether apoptosis simply reflects subendocardial infarction or whether myocyte apoptosis occurs from chronic episodes of reversible myocardial ischemia (ie, angina pectoris). If apoptosis were significantly increased on a chronic basis, it would need to substantially reduce myocyte numbers over time, as has been demonstrated globally in patients with
end-stage ischemic cardiomyopathy, in whom myocyte numbers were reduced in association with an increase in myocyte apoptosis. Because the time course of apoptosis may be short, a modest but chronic increase could effect substantial myocyte loss over time and be an important contributing factor in the progression of coronary artery disease to heart failure in patients who do not have a history of myocardial infarction.

We performed the present study to test the hypothesis that chronic episodes of reversible ischemia induce myocyte loss and regional left ventricular remodeling in viable chronically dysfunctional myocardium. Because it would be difficult to definitively address the effects of apoptosis on myocyte loss in humans because of limited tissue sampling and the presence of factors that could stimulate apoptosis independent of ischemia (ie, increased preload and neurohormonal activation), we examined regional myocyte morphometry in pigs with a severe chronic left anterior descending coronary artery (LAD) stenosis resulting in hibernating myocardium in the absence of heart failure, infarction, and replacement fibrosis.

Methods
Procedures were performed in accordance with institutional guidelines for the care and use of animals in research. The surgical preparation and study protocol have previously been described in detail. Briefly, pigs (8.8 ± 0.4 kg) were sedated with a mixture of 0.037 mL/kg IM of a Telazol-Ketamine mixture (50 mg/mL, tiletamine and 100 mg/mL, ketamine) intubated, and anesthetized with halothane (0.5% to 2%) and oxygen (balance). Eleven pigs (hibernating group) were chronically instrumented with a fixed-diameter (1.5- to 2.0-mm) external stenosis that was secured around the proximal LAD with umbilical tape. Nine pigs (sham group) served as sham-operated controls in which the groove of the occluder was left open to prevent the formation of a significant stenosis. The chest incision was closed, the pneumothorax was evacuated, and analgesia was provided by an intercostal nerve block (2% lidocaine) and butorphanol (0.025 mg/kg). Coronary angiography was performed at baseline and following the occlusion. Forty minutes after occlusion, the LAD was recanalized, and hemodynamic measurements were repeated. The chest incision was closed, and the animals were allowed to recover for 3 weeks, after which they were reanesthetized and perfused with 1% paraformaldehyde and 4% glutaraldehyde for electron microscopy and 10% neutral buffered formalin and embedded in paraffin for light microscopy.

Histological Analysis
After the physiological study, the heart was excised, and blocks of tissue were taken from the central LAD and normal remote zones to assess regional perfusion. Additional samples were fixed in 10% formalin and embedded in paraffin for light microscopy. Myocyte nuclear density was determined from 100 transversely sectioned fields in the subendocardial half of each sample at a magnification of ×600 by use of 5-μm sections stained with hematoxylin and eosin or p-aminosalicylic acid, as previously described by Beltrami et al. Connective tissue was quantified by point counting of trichrome-stained sections, as we have previously described. Connective tissue staining was subtracted from the total tissue area to determine the percentage that was represented by myocytes (percent myocyte area). Histological review was blinded as to whether samples were from sham or hibernating animals but not to location of the sample within the left ventricle.

Quantification of Myocyte Apoptosis by TUNEL and Electron Microscopy
Myocyte apoptosis was detected by terminal deoxynucleotidyl transferase–mediated dUTP nick end-labeling (TUNEL, Chemicon Inc) and epifluorescence with an FITC filter. The inner half of each sample was scanned at ×200, and fluorescing nuclei of myocyte origin were confirmed by examination at high power (×600). TUNEL-positive nuclei that could not be definitively confirmed to be of myocyte origin were excluded. Approximately 300 fields were examined per sample (average LAD area, 30.7 mm² in instrumented pigs and 34.5 mm² in sham-operated pigs). The extent of apoptosis was expressed by normalizing the results to the number of myocyte nuclei per mm² in each sample.

Two additional animals had subendocardial samples evaluated by electron microscopy for necrosis and nuclear chromatin margination and condensation in the presence of intact sarcolemma and mitochondria, which are indicative of apoptosis. Fresh samples were immersion-fixed in glutaraldehyde, postfixed in osmium, and embedded in Embed Araldite mixture by following routine procedures. Ultrathin sections were stained with uranyl acetate and lead citrate. All myocyte nuclei that were available (~500 myocyte nuclei per sample) were evaluated with a JEOL 100CX electron microscope.

Morphometric Calculations
By use of a Zeiss Axioskop microscope and Encore Image Software, myocyte diameter and nuclear length were determined from digitized images of 7-μm sections stained with Sirius red in a subgroup of 7 hibernating pigs. Morphometric analysis of myocyte volume requires the determination of 4 independent parameters. These include the number of myocyte nuclei per unit area, the average length of myocyte nuclei, the average myocyte diameter, and volume fraction of myocytes in the tissue. Regional variations in myocyte volume between LAD and remote regions were assessed by use of standard morphological calculations as previously described in detail by others. Tabulated results represent averages of 25 to 50 longitudinally oriented myocytes from each pair of hibernating and normally perfused remote regions.

All values are mean ± SEM. Statistical comparisons were performed by using 2-tailed t tests with paired comparisons for LAD versus remote zone changes and group comparisons for hibernating versus sham animals. Significance was defined as P < 0.05.

Results
All of the pigs were in good health at the time of study, and blood gases were within physiological limits. None of the animals had evidence of myocardial infarction on gross examination. In addition, there was no gross evidence of regional differences in wall thickness.

Resting hemodynamics were similar between hibernating and sham controls, and the subsequent analysis summarizes average values for all animals studied. There was no clinical or hemodynamic evidence of heart failure in any of the animals. Heart rate averaged 85 ± 3 bpm, and mean aortic pressure was 93 ± 5 mm Hg. Left ventricular end-diastolic pressure was similar in sham (18.6 ± 2.2 mm Hg) and hibernating (17.2 ± 1.2 mm Hg) animals. Although global left ventricular function was normal, anterior wall motion was depressed in hibernating versus sham-instrumented animals (0.5 ± 0.2 versus 2.1 ± 0.2, P < 0.001). Coronary angiography demonstrated total LAD occlusion and collateral-dependent myocardium in all but one of the hibernating animals. Subendocardial flow in the LAD region under resting conditions was significantly reduced in hibernating compared with sham animals (0.65 ± 0.08 versus 0.98 ± 0.14 mL·min⁻¹·g⁻¹, P < 0.05). There was a critical limitation in LAD subendocard-
dial flow reserve, because flow during adenosine failed to increase above the resting values (0.78±0.20 mL ⋅ min⁻¹ ⋅ g⁻¹, P=NS versus rest). In contrast, subendocardial flow increased to 3.24±0.50 mL ⋅ min⁻¹ ⋅ g⁻¹ in the sham group (P<0.001 versus hibernating group).

Regional Myocyte Loss and Apoptosis in Hibernating Myocardium

Figure 1 illustrates a fluorescence TUNEL-positive myocyte and a corresponding bright-field (phase-contrast) photomicrograph at high-power magnification from a representative hibernating animal. TUNEL-positive myocytes occurred in isolation and were never clustered. Bright-field and/or background fluorescence readily identified myocyte structure from other cell types.

Figure 2 summarizes regional variations in myocyte apoptosis, myocyte nuclear density (an index of myocyte numbers), and the percentage of the cross section with myocyte staining (100% minus percent fibrosis). Myocyte apoptosis occurred with increased frequency in dysfunctional LAD regions (220±77 apoptotic myocyte nuclei per 10⁶ myocyte nuclei in hibernating group versus 30±9 apoptotic myocyte nuclei per 10⁶ myocyte nuclei in sham group, P<0.05), although it represented a small fraction of the total myocytes examined (0.022±0.008% in hibernating LAD regions versus 0.003±0.0001% in sham LAD regions, P<0.05). In conjunction with the increase in LAD TUNEL-positive myocytes, hibernating animals exhibited a pronounced reduction in LAD myocyte nuclear density, which averaged 65% of the values in sham controls (995±100 myocytes per mm² in hibernating LAD regions versus 1534±65 myocytes per mm² in sham LAD regions, P<0.005). The reduction in nuclear density was not due to replacement fibrosis, because connective tissue staining was minimally increased (5.8±0.9% in hibernating group versus 3.0±0.2% in sham group, P<0.05). Thus, the percentage of the tissue section that was myocyte area was minimally reduced.

We also quantified apoptosis and myocyte nuclear density in the normally perfused remote regions from pigs with hibernating myocardium (Figure 2). Interestingly, remote regions exhibited an intermediate frequency of apoptosis by TUNEL that was between values obtained in the LAD...
regions from hibernating pigs and sham controls (107±28 apoptotic myocytes per 10^6 myocyte nuclei, P=NS versus sham controls). This was not accompanied by a significant reduction in myocyte nuclear density (1460±89 myocytes per mm^2 in normal remote regions versus 1534±65 myocytes per mm^2 in sham control regions, P=NS) nor was it associated with any change in connective tissue (3.7±0.4% in remote regions versus 3.0±0.2% in sham control regions, P=NS).

We examined subendocardial samples from 2 additional animals to determine whether electron microscopy could identify the low frequency of apoptosis we found. Review of >500 myocyte nuclei in each sample failed to demonstrate evidence of apoptosis by electron microscopy despite profound myocyte loss on light microscopy and apoptosis demonstrated by TUNEL. Of equal importance, electron microscopy also failed to show any changes indicative of myocyte necrosis.

**Regional Myocyte Hypertrophy**

In light of the reduction in myocyte numbers and absence of gross thinning of the anterior wall, we assessed myocyte size by morphometric techniques in a subset of 7 pigs in which tissue blocks provided enough myocytes sectioned longitudinally and in cross section to perform morphometric analyses. Figure 3 shows a high-power photomicrograph of cross-sectionally oriented subendocardial myocytes from the hibernating LAD and normally perfused remote region from an individual animal. In addition to the obvious reduction in myocyte nuclear numbers in the hibernating LAD region, there was a pronounced increase in myocyte size. The Table summarizes paired measurements of myocyte morphometry in hibernating LAD and normal remote regions of the same animal as derived from morphometric analysis. Myocyte nuclear density decreased to 71% of values in remote regions. The volume fraction of myocytes in hibernating myocardium was maintained by regional hypertrophy as manifested by a 55% increase in myocyte volume. Thus, gross wall thinning from myocyte loss was prevented by regional myocyte hypertrophy.

**Discussion**

The major and important new finding from the present study is that areas of the heart subjected to chronic reversible ischemia develop regional myocyte loss in association with the physiological findings of hibernating myocardium. Regional myocardial wall thinning was prevented by compensatory myocyte hypertrophy that was confined to the viable...
dysfunctional region. Chronic myocyte loss appeared to occur solely through scattered myocyte apoptosis, which was increased to values 7-fold higher than in sham controls. Necrosis was not present, and myocardial connective tissue in hibernating myocardium was minimally increased. This constellation of findings suggests that reversible ischemia associated with chronic coronary disease has the potential to effect significant regional myocyte loss and remodeling over time. The regional myocyte loss and compensatory hypertrophy may be an important mechanism leading to the late development of ischemic cardiomyopathy. It may also account for the frequently observed incomplete recovery of function in hibernating myocardium after revascularization.

The presence of apoptosis in humans with hibernating myocardium is controversial. Elsässer et al found “several cases” of apoptotic myocytes by electron microscopy of needle biopsies from patients with reversibly dyssynergic myocardium. The limited amount of biopsy material made it impossible to perform a systematic analysis to quantify the frequency of apoptosis or myocyte loss. In addition, myocyte morphometry could not be quantified. Apoptosis was predominantly present in a subgroup of patients in which fibrosis averaged 58% of the biopsy (stage 3 degeneration). These regions exhibited the least amount of functional recovery after revascularization, leading the authors to speculate that hibernating myocardium may inexorably progress to an advanced degenerative state requiring revascularization to prevent structural fibrosis. In contrast, electron microscopic nuclear condensation typical of apoptosis was not seen by Ausma et al in patients with hibernating myocardium, in which fibrosis averaged only 11% of the biopsy. One explanation for the discordant clinical reports is that apoptosis is present only with advanced fibrosis. Thus, it might actually reflect changes typical of a recent or healing subendocardial infarction. Another more likely explanation is that the tissue sampling from a full-thickness needle biopsy (having limited subendocardial myocytes) was insufficient to detect a low but chronically increased rate of apoptosis in viable dysfunctional myocardium.

Our results help to resolve some of these discordant clinical findings. Hibernating myocardium in pigs was not associated with subendocardial infarction, and necrosis was not seen by light or electron microscopy. Connective tissue was minimally increased over sham-operated and remote regions and was similar to values reported in humans with chronic coronary disease and normal or hibernating myocardium. Apoptosis by TUNEL staining was rare and present in single scattered myocytes. We surveyed a much larger cross-sectional area of myocardium (30 to 35 mm²) than available on a needle biopsy and identified fluorescenting myocyte nuclei by TUNEL staining in ≈1 of 5000 myocyte nuclei. This low frequency was undoubtedly the reason that we, like Ausma et al, failed to identify any apoptotic myocyte nuclei by electron microscopy. Thus, electron microscopy is a relatively insensitive approach to identify or quantify apoptosis in this chronic situation.

The constellation of apoptosis, myocyte loss, and compensatory hypertrophy has been found in humans with end-stage congestive heart failure from ischemic cardiomyopathy. It has, however, been difficult to determine the extent to which these changes reflect the effects of regional ischemia, myocardial infarction, myocyte stretch, and neurohormonal activation, which can all modulate myocardial remodeling independently. In the present study, a critical limitation in flow reserve resulted in regional apoptosis in the absence of infarction or fibrosis. We have previously documented that the physiological features of this model are similar to those of humans with hibernating myocardium and include regionally reduced resting flow and function, recruitable inotropic reserve, and increased 18F-2-deoxyglucose uptake in the fasting state. The regional dysfunction was not associated with heart failure, and left ventricular filling pressure was similar in hibernating and sham-instrumented animals. Thus, our findings support the notion that repetitive episodes of ischemia are an independent stimulus that leads to myocyte apoptosis. Because ischemic cardiomyopathy is usually associated with multivessel coronary disease, the possibility exists that the progression of heart failure may be related to reversible ischemic injury and apoptosis with irreversible replacement fibrosis occurring at a relatively late stage of the disease.

A problem in interpreting the importance of myocyte apoptosis in acute studies has been quantifying its overall impact on myocyte loss. Although we were unable to identify apoptosis by electron microscopy, the measurements of regional myocyte nuclear density provided a direct evaluation indicating that the long-term impact of apoptosis was very significant. We found myocyte nuclear density in hibernating myocardium to be reduced to values that were 65% of values in sham controls and 68% of values in remote, normally perfused regions from the same hearts. Rates of apoptosis vary widely among studies and are particularly high after acute or short-term proapoptotic stimuli. Although we demonstrated significant regional myocyte loss, the frequency of apoptosis was considerably lower than the 9.8% frequency of subendocardial apoptosis reported in acute ischemic injury associated with patchy necrosis in pigs subjected to a period of short-term hibernation. As pointed out by others, a chronic process with rates this high would quickly be incompatible with life unless TUNEL positivity of individual myocytes lasted for a period of several weeks, which is unlikely (see below). We also found apoptosis in single myocytes that were widely dispersed, in contrast to the clustered distribution seen with prolonged acute ischemia or acute infarction. It is likely that apoptosis varies in relation to the frequency and/or severity of ischemia as well as to other unidentified factors. Because we examined only one time point, we cannot exclude the possibility that apoptosis may have been higher at times before the animals with hibernating myocardium were euthanized. Nevertheless, a preliminary report examining apoptosis at 1 and 2 months demonstrated a lower frequency of myocyte apoptosis in viable dysfunctional myocardium that increased after 1 month of instrumentation.
The duration of the nick translation assay positivity in cardiomyocytes before death and disappearance in vivo is unknown. Some studies have suggested that apoptosis is completed in as little as 20 minutes, whereas others indicate that it could last as long as 24 hours. \(^8,10\) We can make an estimate of the duration that myocytes are TUNEL positive in vivo based on the findings of the present study and several assumptions. First, for simplicity, we assume that this occurs as a constant process between 30 and 95 days after instrumentation and that the frequency of apoptosis at any given time averages 0.022%. If we assume that apoptosis begins after 1 month and that the 35% reduction in nuclear density occurs over 65 days, an average of 0.54% of total myocytes will be lost every 24 hours. The duration of TUNEL-positive staining can be estimated by the proportionality (0.54% myocytes/24 hours = 0.022%/duration of apoptosis), yielding \(\approx 1\) hour. This is within the time frame reported in other studies and also points out the significance of a chronic low-grade process. If the duration had been 24 hours, the frequency of apoptosis in hibernating myocardium would have had to be \(\approx 0.5\%\), which was well above the highest frequency that we observed in an individual animal (0.08%).

Our data also show that myocyte apoptosis is increased to intermediate levels in the normally perfused remote regions of hearts with viable dysfunctional myocardium (Figure 2). Although we were unable to show that this was associated with a significant reduction in myocyte nuclear density, there was a trend for values to be lower than those in myocardium from sham controls. The exact stimuli responsible for this finding remain to be established, but several possibilities deserve consideration. Although right heart catheterization was not performed, none of the animals had evidence of congestive heart failure, and systemic hemodynamics were similar in each group. Thus, neurohormonal activation is probably an unlikely explanation for increased apoptosis. Although the ventricular end-diastolic pressure was mildly increased because of the halothane anesthetic regimen at the time we studied the animals, it was similar in sham and hibernating groups. Although this makes stretch-induced apoptosis\(^17\) a less likely explanation, pigs with a chronic stenosis undoubtedly developed episodes of elevated left ventricular end-diastolic pressure during exercise-induced ischemia in the presence of a critical coronary stenosis. Thus, it is possible that transient reversible increases in preload were responsible for the findings. A final possibility may be that the large viable dysfunctional region stimulates apoptosis through mechanisms that are similar to those in left ventricular remodeling.\(^18\) This could arise in the absence of heart failure and increased preload through discontinuities in the distribution of stress in the functional border zone between normally perfused and chronically dysfunctional regions. Remote zone apoptosis may ultimately prove to be a determinant of the progression of chronic ischemic heart disease to heart failure, although further studies will be required to determine its mechanisms.

**Methodological Limitations**

We could not confirm the specificity of TUNEL to detect apoptosis with an independent technique because the frequency was insufficient to identify by electron microscopy. Although necrosis was also not identified by light or electron microscopy, we cannot exclude the possibility that the TUNEL positivity is related to oncosis as opposed to apoptosis.\(^19\) This would not affect our major findings because, regardless of the mechanism of cell death, the regional reduction in myocyte nuclear density directly demonstrates cell loss without fibrosis in hibernating myocardium.

In summary, our results indicate that ischemia from a chronic stenosis can effect substantial myocyte loss in the absence of significant fibrosis. How this regional myocyte loss ultimately impacts on the recovery of ventricular function after revascularization of hibernating myocardium and the progression of ischemic heart disease remains undefined. Based on these findings, aggressive medical and surgical interventions designed to ameliorate ischemia may have a substantial impact in halting apoptosis and the progression of coronary disease to ischemic cardiomyopathy.

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