Amlodipine inhibits granulation tissue cell apoptosis through reducing calcineurin activity to attenuate postinfarction cardiac remodeling

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Ogino A, Takemura G, Kanamori H, Okada H, Maruyama R, Miyata S, Esaki M, Nakagawa M, Aoyama T, Ushikoshi H, Kawasaki M, Minatoguchi S, Fujiwara T, Fujiwara H. Amlodipine inhibits granulation tissue cell apoptosis through reducing calcineurin activity to attenuate postinfarction cardiac remodeling. Am J Physiol Heart Circ Physiol 293: H2271–H2280, 2007. First published July 20, 2007; doi:10.1152/ajpheart.00303.2007.—Although amlodipine, a long-acting L-type calcium channel blocker, reportedly prevents left ventricular remodeling and dysfunction after myocardial infarction, the mechanism responsible is not yet well understood. Myocardial infarction was induced in mice by ligating the left coronary artery. Treatment of mice with amlodipine (10 mg·kg−1·day−1), beginning on the third day postinfarction, significantly improved survival and attenuated left ventricular dilatation and dysfunction 4 wk postinfarction compared with treatment with saline or hydralazine. Although infarct sizes did not differ among the groups, the infarcted wall thickness was greater and the infarct segment length was smaller in the amlodipine-treated group, and cellular components, including vessels and myofibroblasts, were abundant within the infarcted area. Ten days postinfarction (the subacute stage), the proliferation of granulation tissue cells in the infarcted area was similar among the groups, but the incidence of apoptosis was significantly lower in the amlodipine-treated group, where Bad, a proapoptotic Bcl-2 family protein, was significantly phosphorylated (inactivated). Calcineurin, which dephosphorylates (activates) Bad, was upregulated in infarcted hearts, but its levels were significantly reduced by amlodipine treatment. In vitro, Fas stimulation augmented calcineurin activity and induced apoptosis among infarct tissue-derived myofibroblasts; both of those effects were strongly inhibited by amlodipine, two other calcium channel blockers (verapamil or nifedipine), and two calcineurin inhibitors (cyclosporin A or FK-506). Amlodipine inhibits Fas-mediated granulation tissue cell apoptosis in infarcted hearts, possibly by attenuating the activities of calcineurin and Bad. These findings may provide new insight into the mechanism by which calcium channel blockers attenuate postinfarction cardiac remodeling and dysfunction.

Ca2+ channels; heart failure

CALCIUM CHANNEL BLOCKERS (CCBs) are widely used in the treatment of hypertension and ischemic heart diseases because of their systemic and coronary vasodilating effects (2, 14). Amlodipine, a so-called third-generation CCB, appears to have fewer negative inotropic effects than earlier CCBs and improves the prognosis of patients (1). In addition, with its slow onset of action and long plasma half-life (36 h), amlodipine is unlikely to strongly activate the neurohumoral system and reportedly improves the prognosis of patients with nonspecific heart failure (15). In experimental settings, amlodipine has been shown to diminish cardiac remodeling in spontaneously hypertensive rats (28), rats with myocardial infarctions (MIs) (20, 23), and hamsters with dilated cardiomyopathy (27). The precise mechanisms for these beneficial effects are not yet fully understood, although the prevention of phenotypic changes in sarcomeric proteins and anti-inflammatory, antioxidant, and antifibrotic effects have all been suggested.

Large MIs cause severe chronic heart failure with unfavorable remodeling of the left ventricle (LV) that is characterized by LV dilatation and diminished cardiac performance (17). Although the magnitude of the acute MI, which can be determined within several hours of an attack (18), is the most critical determinant of subsequent heart failure, many other factors, including cardiomyocyte hypertrophy, fibrosis, and the expression of various cytokines, also are associated with disease progression (22). This is because infarcted tissue is highly dynamic and shows remarkable changes during the course of healing: necrotic tissue is infiltrated by inflammatory cells during the acute stage of MI; granulation tissue forms during the subacute stage; and scar tissue forms during the chronic stage (24, 29). Most cellular components that infiltrate and proliferate within an infarct, including inflammatory and granulation tissue cells, disappear via apoptosis during the subacute and acute stages (25). In that regard, we (5, 8) have previously reported that granulation tissue cell apoptosis is Fas and caspase dependent and that its inhibition during the subacute stage of MI alters infarct tissue dynamics, resulting in the mitigation of adverse LV remodeling and dysfunction at the chronic stage.

Intracellular calcium levels are critically important for the execution of apoptosis, and inhibition of calcium influx is known to prevent apoptosis in many cell types (12). For instance, we and others (9, 19) have reported that the CCB nifedipine prevents β-adrenergic-mediated apoptosis among cardiac myocytes by inhibiting the elevation of intracellular calcium levels. We therefore hypothesized that CCBs might alter infarct tissue dynamics by preventing apoptosis, thereby influencing postinfarction LV remodeling and function. To test that idea, in the present study, we first performed a functional and morphological analysis of calcium-channel-mediated alterations in post-MI hearts by inhibiting calcium influx using the CCB amlodipine. We then sought to elucidate the patho-
physiological and molecular mechanism underlying the ob-
served effects, focusing in particular on the antiapoptotic effect of CCBs. Since as well as cell death, cell proliferation is
another critical determinant for tissue dynamics, we also ex-
aimed the effect of amlodipine on proliferating activity in the
infarct tissue.

MATERIALS AND METHODS

In Vivo Animal Experiments

This study was approved by our Institutional Animal Research
Committee. MI was induced in 9-wk-old male C57BL/6J mice
(Chubu Kagaku) by ligating the left coronary artery (n = 100) as
previously described (8). In sham-operated mice (n = 11), the suture
was passed around the artery but not tied. On the third day after
surgery, 81 mice in the MI group remained alive (survival rate: 81%),
whereas all mice in the sham-operated group survived. The 81
MI-bearing mice were randomly assigned to 3 groups: a saline group
(n = 27), which served as the untreated control group; a hydralazine
group (n = 27, 15 mg·kg\(^{-1}\)·day\(^{-1}\), Sigma), which served as the
hypotension control group; and an amlodipine group (n = 27, 10
mg·kg\(^{-1}\)·day\(^{-1}\), a gift from Pfizer). Prior to the initiation of the
treatment protocols, all three groups showed similar cardiac function
on echocardiography (Table 1). The reagents were administered using
subcutaneously embedded osmotic minipumps (Alzet) beginning on
the third day after surgery and continuing until death. The osmotic
pumps were used to assure a stable administration of the chemicals.
Animals were killed 4 wk after surgery.

In another set of experiment, mice were sham operated or MI
induced and then treated with each reagent in the same manner as
described above. They were killed 10 days after surgery, after which
hearts were used for histological, immunohistochemical, and bi-
chemical assays.

Cell Culture and Treatment

Ten days after the induction of MI in mice, cardiac myofibroblasts
were collected from the infarcted areas of the hearts as previously
described (7) with some modifications. Briefly, the hearts were re-
sected, and the infarcted areas were removed. The tissue was then
minced and incubated with collagenase type II (Worthington) in
Kreb's-Ringer buffer for 30 min at 37°C. The dissociated cells were
plated on 10-cm dishes for 1 h and then rigorously washed with
buffer. The attached nonmyocytes remaining were cultured in DMEM
supplemented with 5% FBS, and >90% were found to be α-smooth
muscle actin (α-SMA) positive. These cells were used for experiment-
tation at the second passage. The medium was replaced with serum-
free DMEM for 24 h, after which a mixture of agonistic anti-Fas
antibody (1 μg/ml, Pharmingen) and actinomycin D (0.05 μg/ml,
Sigma) was applied to the cells for 24 h to induce apoptosis. Fas
stimulation with these reagents has been established to cause apop-
tosis in this cell type (13). In other experiments, ionomycin (1 μmol/L,
Sigma) instead of anti-Fas antibody plus actinomycin D was applied
to the cells for 24 h to induce cell death. In some experiments,
the cells were pretreated with amlodipine, another CCB [nifedipine
(1 μmol/L, Sigma) or verapamil (100 nmol/L, Wako)], or a calcineurin
inhibitor [cyclosporin A (100 nmol/L, Wako) or FK-506 (100 ng/ml,
Sigma)] prior to the addition of the anti-Fas antibody and actinomycin
D or ionomycin. Each in vitro experiment was done at least in triplicate.

Physiological Experiments

Blood pressure and heart rate were measured before and 2 and 4 wk
after surgery using the tail-cuff method (BP MONITOR, Muromachi
Kikai). Physiological experiments (echocardiography and cardiac
catheterization) were carried out as previously described (8) with
modifications. Animals were anesthetized with halothane (induction:
2%; maintenance: 0.5%) in a mixture of N\(_2\)O and O\(_2\) (0.5 l/min each)
via a nasal mask. Echocardiograms were recorded using an echocar-
diographic system (Vevo770, Visualsonics) equipped with a 45-MHz
imaging transducer before treatment and at death. Following echocar-
diography, the right carotid artery was cannulated with a microm-
rometer-tipped catheter (SPR 671, Millar Instruments) that was ad-
vanced into the aorta and then into the LV for recording pressure and
maximal and minimal dP/dt (±dP/dt).

Histology

Once the physiological measurements were complete, all surviving
mice were killed, and the hearts were removed. Eight hearts from each
group were randomly chosen for histological analysis, and they were
cut into two transverse slices through the middle of the infarct,
between the atrioventricular groove and the apex. Basal specimens
were fixed in 10% buffered formalin, embedded in paraffin, cut into
sections of 5-μm thicknesses, and stained with hematoxylin-eosin, Mason’s
trichrome, and Sirius red F3BA (0.1% solution in saturated aqueous
picric acid, Aldrich). Quantitative assessments, including cell sizes
and cell numbers, were carried out in randomly chosen high-power
fields (HPFs) in each section; morphometric analyses of the areas of
the infarct, fibrosis, and immunopositive cells were carried out using

Table 1. Cardiac function during the physiological examination carried out before treatments and 4 wk after surgery

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>Saline</th>
<th>Hydralazine</th>
<th>Amlodipine</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>11</td>
<td>15</td>
<td>20</td>
<td>25</td>
</tr>
<tr>
<td>LVEd, mm</td>
<td>3.51 ± 0.06</td>
<td>3.90 ± 0.06*</td>
<td>3.88 ± 0.05*</td>
<td>3.92 ± 0.03*</td>
</tr>
<tr>
<td>LVEF, %</td>
<td>73.9 ± 1.1</td>
<td>58.7 ± 1.0*</td>
<td>58.5 ± 0.8*</td>
<td>59.9 ± 0.9*</td>
</tr>
<tr>
<td>4 wk after surgery</td>
<td>3.55 ± 0.08</td>
<td>5.45 ± 0.18*</td>
<td>5.42 ± 0.09*</td>
<td>4.44 ± 0.08**†‡</td>
</tr>
<tr>
<td>LVEd, mm</td>
<td>72.0 ± 1.2</td>
<td>33.1 ± 3.1*</td>
<td>36.4 ± 1.6*</td>
<td>47.0 ± 2.5**‡‡</td>
</tr>
<tr>
<td>LVEF, %</td>
<td>73.9 ± 1.1</td>
<td>50.2 ± 2.3</td>
<td>490.7 ± 1.7</td>
<td>505 ± 1.3</td>
</tr>
<tr>
<td>Heart rate, beats/min</td>
<td>92.9 ± 9.5</td>
<td>69.7 ± 3.7*</td>
<td>77.4 ± 2.0*</td>
<td>77.4 ± 2.0*</td>
</tr>
<tr>
<td>MBP, mmHg</td>
<td>81.1 ± 7.2</td>
<td>8.9 ± 1.0*</td>
<td>5.6 ± 0.7*</td>
<td>2.8 ± 0.5*‡‡</td>
</tr>
<tr>
<td>LVEDP, mmHg</td>
<td>8.0 ± 0.3</td>
<td>8.9 ± 1.0*</td>
<td>5.6 ± 0.7*</td>
<td>2.8 ± 0.5*‡‡</td>
</tr>
<tr>
<td>+dP/dt, mmHg/s</td>
<td>9,758 ± 908</td>
<td>3,873 ± 406*</td>
<td>4,096 ± 245*</td>
<td>4,096 ± 245*</td>
</tr>
<tr>
<td>−dP/dt, mmHg/s</td>
<td>−3,186 ± 943</td>
<td>−3,370 ± 527*</td>
<td>−3,392 ± 267*</td>
<td>−6,013 ± 262*†‡‡</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of animals. MI, myocardial infarction; LVEd, end-diastolic left ventricular (LV) diameter; LVEF, LV ejection fraction; SBP, systolic blood pressure; MBP, mean blood pressure; LVEDP, LV end-diastolic pressure; dP/dt, change in pressure over time. *P < 0.05 vs. the sham group; †P < 0.05 vs. the saline group; ‡P < 0.05 vs. the hydralazine group.
a multipurpose color image processor (LUZEX F, Nireco). The size of the MI and the fibrotic area in the noninfarcted region were measured by searching the entire ventricle. Cardiomyocyte size (expressed as the transverse diameter of myocytes cut at the level of the nucleus) and cell populations were assessed in 20 randomly chosen HPFs in each section.

**Immunohistochemistry**

After deparaffinization, 4-μm-thick sections were incubated with a primary antibody against α-SMA (1A4, Sigma), von Wilbrand factor (vWF; Dako), macrophages (F4/80, UK-Serotec), or Ki-67 (Santa Cruz Biotechnology). A Vectastain Elite ABC system (Vector Laboratories) was then used to immunostain the sections; diaminobenzidine served as the chromogen, and the nuclei were counterstained with hematoxylin. Quantitative assessments, including the numbers and areas of immunopositive cells, were made in 20 randomly chosen HPFs using a multipurpose color image processor.

In situ TUNEL assays were carried out with sections using an ApopTag kit (Chemicon) according to the manufacturer’s instructions. Mouse mammary tissue served as a positive control.

For double-label immunofluorescence and TUNEL with α-SMA, vWF, or F4/80, sections were respectively labeled using Fluorescein- or Texas Red-conjugated secondary antibody (Molecular Probes). Sections were then counterstained with Hoechst 33342 and observed under a confocal microscope (LSM510, Zeiss).

**Western Blot Analysis**

Proteins (100 μg) extracted from hearts (n = 4–6 from each group) were subjected to 14% polyacrylamide gel electrophoresis and then transferred onto polyvinylidene difluoride membranes. Membranes were then probed using primary antibodies against Fas and Fas ligand (both from BD Transduction Laboratories/BD Pharmingen); Fas-associated death factor (FADD) and Daxx (both from Santa Cruz Biotechnology); caspase-8 and caspase-3 (both from Cell Signaling); Bcl-2, Bax, and Bcl-xL (all from Santa Cruz Biotechnology); Bad (Cell Signaling) and the phosphorylated form (Ser136) of Bad (Cell Signaling); and JNK and the phosphorylated form of JNK (p-JNK; both from Santa Cruz Biotechnology). Blots were then visualized using enhanced chemiluminescence (Amersham). β-Tubulin (antibody from Santa Cruz Biotechnology) served as the loading control.

To detect cytochrome c, cells were suspended in a buffer [20 mM HEPES (pH 7.5), 10 mM KCl, 1.5 mM MgCl2, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and 1 mM PMSF] for 3 min on ice, homogenized by 10 strokes in a Dounce homogenizer, and centrifuged at 15,000 rpm for 15 min. The supernatant was the cytosol fraction, and the pellet was resolved in lysis buffer as the membrane fraction. The antibody used was anti-cytochrome c polyclonal antibody from Santa Cruz Biotechnology.

**Calcineurin Assay**

After various treatments, the whole ventricles or cultured myofibroblasts derived from 10-day-old infarct tissue were collected, and total proteins were extracted by lysing the cells in lysis buffer [50 mM Tris-HCl (pH 7.5), 10 mM EDTA, 100 mM NaCl, and 0.1% Triton X-100]. Phosphate-reduced samples were then prepared, and calcineurin (also known as protein phosphatase 2B) activity was detected using a serine/threonine Phosphatase Assay System according to the manufacturer’s instructions (Promega). After the addition of calmodulin to the assay mixture, the amount of free phosphate released from a phosphopeptide substrate by calcineurin was measured using molybdate dye solution, and enzyme activity was expressed as a percentage of the control. Experiments were done at least in triplicate in each group.

**Statistical Analysis**

Data are expressed as means ± SE. Survival was analyzed using the Kaplan-Meier method using the log-rank Cox-Mantel method. The significance of differences between groups was evaluated using one-way ANOVA with a post hoc Newman-Keul’s multiple-comparisons test. Values of P < 0.05 were considered significant.

**RESULTS**

**Effect of Amlodipine on Survival and Cardiac Remodeling and Function 4 wk Post-MI**

Systolic blood pressures did not significantly differ between mice in the hydralazine and amlodipine groups, both of which were lower than in the saline group (Fig. 1A). Heart rates were similar among all the groups (Fig. 1A). Rate-pressure products were lower in the hydralazine and amlodipine groups, between which there was no difference (Fig. 1A).

All sham-operated mice (n = 11) survived 4 wk after the surgery. Among the MI-bearing groups, the survival rate was...
significantly greater in the amlodipine group (93%, 25 survived of 27 mice) than in the saline group (56%, 15 survived of 27 mice), but there were no significant differences between the hydralazine (74%, 20 survived of 27 mice) and saline groups (Fig. 1B).

Echocardiography and cardiac catheterization carried out 4 wk post-MI revealed that compared with sham-operated mice, control MI-bearing mice showed severe LV remodeling with marked enlargement of the LV cavity and signs of diminished cardiac function, i.e., significantly reduced LV ejection fraction, reduced ±dP/dt, and increased LV end-diastolic pressure (Table 1). Each of these conditions was significantly attenuated in amlodipine-treated mice but not in hydralazine-treated mice.

**Histology of Hearts 4 wk Post-MI**

**Gross morphometry.** Heart weight-to-body weight ratios were significantly smaller in the amlodipine group (5.0 ± 0.17 mg/g) than the control groups (saline: 6.3 ± 0.40 mg/g and hydralazine: 6.1 ± 0.17 mg/g). Whereas hearts from saline- and hydralazine-treated mice showed marked LV dilatation with a thin infarcted segment, those from amlodipine-treated mice showed substantially smaller LV cavities and thicker infarcted segments with shorter circumferential lengths (Fig. 2A and Table 2). On the other hand, both the absolute area of the infarct and the percentage of the LV taken up by the infarct were comparable among all groups (Table 2).

**Infarcted area.** By 4 wk post-MI, the infarcted area had been replaced by fibrous scar tissue in saline- and hydralazine-treated mice. In amlodipine-treated mice, in contrast, the infarcted area contained not only collagen fibers but also abundant cellular components. The overall population of noncardiomyocyte interstitial cells within the infarct area was significantly larger in amlodipine-treated mice (Fig. 2B and Table 2). In addition, the percent area of extravascular α-SMA-positive cells and the numbers of vWF-positive blood vessels present within the infarcted area also were greater in amlodipine-treated mice than in the other two groups (Fig. 2, C and D, and Table 2). Few macrophages were observed in any of the groups (Fig. 2E and Table 2).

**Noninfarcted area.** Sirius red staining showed there to be significantly less fibrosis in noninfarcted LV walls of amlodipine-treated hearts than in those of saline- and hydralazine-treated hearts (Fig. 2F and Table 2). In addition, the transverse diameters of the cardiomyocytes in the noninfarcted areas were significantly greater in the saline and hydralazine groups than in the amlodipine group (Table 2), suggesting that compensatory cardiomyocyte hypertrophy was less developed in the amlodipine group.

**Granulation Tissue Cell Dynamics 10 days Post-MI**

As mentioned above, we observed greater numbers of cells, especially α-SMA-positive and endothelial cells, within the infarcted areas of amlodipine-treated hearts during the chronic stage of MI (4 wk post-MI). Because we hypothesized that altered cell dynamics during the ongoing healing process might explain the difference in the composition of the infarct tissue during the chronic stage, we next evaluated apoptosis and proliferation among granulation tissue cells in saline-treated (n = 8), hydralazine-treated (n = 8) and amlodipine-treated (n = 8) hearts 10 days post-MI (subacute stage), which is when granulation tissue peaks in mice.

TUNEL assays revealed apoptosis among granulation tissue cells in hearts from all three groups (Fig. 3A). Notably, however, the incidence of TUNEL positivity was significantly smaller in the amlodipine group than in the saline and hydralazine groups. In addition, although the expression of pro-caspase-3, one of the final effector caspases, was upregulated equally in all of the hearts collected 10 days post-MI, there was significantly less expression of the active forms of caspase-3 (17 and 19 kDa) in the amlodipine group than in the other groups (Fig. 3B). Also, the release of cytochrome c into the cytosolic fraction was significantly reduced in hearts treated with amlodipine (Fig. 3C). Double-immunohistochemical
techniques revealed that treatment with amlodipine significantly reduced the TUNEL-positive rate of endothelial cells and myofibroblasts but not that of macrophage (Fig. 3C). In contrast, immunohistochemical detection of Ki-67 antigen showed similar levels of proliferation among granulation tissue cells in all three groups (Fig. 3E). The noncardiomyocyte population in the infarct area was significantly greater in amlodipine-treated mice than in saline- or hydralazine-treated mice. No Ki-67-positive cardiomyocytes were detected either.

Apoptosis-Related Molecular Signaling in Granulation Tissue Cells

We (9) have previously reported that apoptosis among post-MI granulation tissue cells is, at least in part, Fas dependent. Two distinct signal transduction pathways (classic and alternate) have been identified downstream of the Fas receptor (6, 30). In the classic pathway, FADD is recruited via the death domain of the Fas receptor to activate caspase-8, whereas in the alternate pathway, the Fas binding protein Daxx mediates the activation of apoptosis signal-regulating kinase-1, JNK, and/or p38 (6). Bearing that in mind, we next used Western blot analysis to investigate apoptosis-related molecular signaling, especially via Fas pathways, in granulation tissue collected 10 days post-MI. We found that the expressions of both Fas and Fas ligand were significantly upregulated in granulation tissue from hearts in all three treatment groups and that neither were affected by any of the treatments (Fig. 4A). The level of FADD expression was similar in sham-operated and infarcted hearts, whereas Daxx expression was significantly upregulated in infarcted hearts and was not affected by the treatments (Fig. 4B). The level of caspase-8 activation, which was reflected by the level of cleaved caspase-8 (45 kDa), was clearly increased in infarcted hearts and was similar among all three treatment groups, as was the level of JNK activation (Fig. 4, C and D). In summary, amlodipine did not specifically affect any of the downstream mediators of Fas signaling.

The execution of apoptosis is largely determined by the relative balance between proapoptotic molecules such as Bax and Bad and antiapoptotic molecules such as Bcl-2 and Bcl-xL (21, 32). Bad promotes cell death by inhibiting Bcl-2 and Bcl-xL function, and its inactivation by phosphorylation at Ser136 enhances cell survival. The calcium-dependent phosphatase calcineurin reportedly dephosphorylate Bad and enhance heterodimerization with Bcl-xL, leading to apoptosis (26). In contrast, protein kinase B and MAPK cascades promote cell survival by phosphorylating Bad at Ser136 (3, 21). Western blot analysis revealed that Bax, Bad, Bcl-2, and Bcl-xL were all upregulated in infarcted hearts and that the levels of all these molecules were similar in saline, hydralazine, and amlodipine groups (Fig. 5A). In addition, the level of phosphorylated (inactivated) Bad was significantly lower in infarcted hearts than sham-operated hearts, but levels were significantly restored by amlodipine treatment (Fig. 5A). Consistent with that finding, we detected higher levels of calcineurin activity in infarcted hearts than in sham-operated hearts, but calcineurin activity was not significantly affected by any of the treatments (Fig. 5A).

### Table 2. Effects of treatment of mice with saline, hydralazine, or amlodipine on physical characteristics and cardiac morphometry and histology 4 wk post-MI

<table>
<thead>
<tr>
<th>Physical characteristics</th>
<th>Saline</th>
<th>Hydralazine</th>
<th>Amlodipine</th>
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</thead>
<tbody>
<tr>
<td>n</td>
<td>15</td>
<td>20</td>
<td>25</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>27.3±0.5</td>
<td>26.2±0.3</td>
<td>26.4±0.5</td>
</tr>
<tr>
<td>Heart weight, mg</td>
<td>17.0±0.5</td>
<td>16.1±0.6</td>
<td>13.2±0.6†</td>
</tr>
<tr>
<td>Heart weight/body weight, mg/g</td>
<td>0.63±0.04</td>
<td>0.61±0.02</td>
<td>0.50±0.02†</td>
</tr>
<tr>
<td>Tibial length, mm</td>
<td>26.3±0.3</td>
<td>26.4±0.2</td>
<td>26.5±0.2</td>
</tr>
<tr>
<td>Heart weight/tibial length, mg/mm</td>
<td>0.65±0.04</td>
<td>0.61±0.02</td>
<td>0.50±0.02†</td>
</tr>
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<table>
<thead>
<tr>
<th>Infarcted area</th>
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<tbody>
<tr>
<td>n</td>
<td>8</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>%MI area in a total LV area</td>
<td>26.5±1.3</td>
<td>25.2±1.4</td>
<td>24.0±3.5</td>
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<td>MI length, mm</td>
<td>11.1±0.9</td>
<td>10.4±0.5</td>
<td>7.7±0.8†</td>
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<tr>
<td>%MI segmental length in a total LV circumference</td>
<td>52.8±4.0</td>
<td>49.8±4.6</td>
<td>48.8±3.5</td>
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<tr>
<td>MI wall thickness, ×10⁻² mm</td>
<td>13.9±3.1</td>
<td>18.5±2.7</td>
<td>38.3±6.9*</td>
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<tr>
<td>Cell populations, cells/mm²</td>
<td>147.8±5.3</td>
<td>155.5±3.7</td>
<td>184.4±5.4†</td>
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<td>α-SMA, %positive area</td>
<td>2.41±0.06</td>
<td>2.50±0.17</td>
<td>4.83±0.42†</td>
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<td>vWF vessels, vessels/mm²</td>
<td>4.36±0.16</td>
<td>4.52±0.29</td>
<td>8.90±0.21†</td>
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<td>Macrophages, cells/mm²</td>
<td>2.03±0.13</td>
<td>2.00±0.21</td>
<td>2.22±0.08</td>
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<tr>
<td>Noninfarcted area</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>8</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Non-MI wall thickness, ×10⁻² mm</td>
<td>91.6±3.3</td>
<td>92.7±5.9</td>
<td>72.7±5.1†</td>
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<td>Myocyte size, μm</td>
<td>17.8±0.3</td>
<td>17.6±0.4</td>
<td>15.5±0.5†</td>
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<td>%Sirius red</td>
<td>2.93±0.08</td>
<td>2.96±0.10</td>
<td>1.65±0.06†</td>
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</table>

Values are means ± SE; n, no. of animals. α-SMA, α-smooth muscle actin; vWF, von Willebrand factor. *P < 0.05 vs. the saline group; †P < 0.05 vs. the hydralazine group.
Fig. 3. Apoptosis and proliferation within granulation tissue 10 days post-MI. A: photomicrographs showing TUNEL-positive cells (left) with a graph comparing the incidences of TUNEL positivity in each group (right). Bars = 20 μm. B: Western blot analysis showing procaspase-3 and active caspase-3. C: Western blot analysis showing cytochrome c in mitochondrial and cytosolic fractions. D: graphs showing the incidences of TUNEL positivity separately evaluated in endothelial cells, myofibroblasts, and macrophages. E: photomicrographs showing Ki-67-positive cells (left) and a graph comparing the incidences of proliferating cells in each group (right). Bars = 20 μm. F: graphs showing a total noncardiomyocyte population, vessel density, area of myofibroblasts, and macrophage population in granulation tissues in each group. HPF, high-powered field. *P < 0.05 vs. the sham group; #P < 0.05 vs. the saline group; +P < 0.05 vs. the hydralazine group.
activity was significantly suppressed by amlodipine treatment (Fig. 5B).

Effect of Amlodipine on Fas-Induced Apoptosis Among Cultured Myofibroblasts

Incubation of cultured myofibroblasts derived from granulation tissue with agonistic anti-Fas antibody plus actinomycin D for 24 h induced apoptosis in 7.3 ± 0.4% of the cells (Fig. 6A). Pretreatment with amlodipine suppressed this apoptosis in a dose-dependent manner (Fig. 6B). Pretreatment with nifedipine (1 μmol/l) or verapamil (100 nmol/l) also suppressed it (Fig. 6C). Interestingly, the calcineurin inhibitors cyclosporin A (100 nmol/l) and FK-506 (100 ng/ml) also each inhibited Fas-induced apoptosis among myofibroblasts (Fig. 6C). To further confirm that the observed increase in apoptosis reflected calcium-dependent activation of calcineurin, we examined the effects of the calcium ionophore ionomycin, which causes sustained elevation of the intracellular calcium concentration. We found that ionomycin (1 μmol/l) induced cell death among myofibroblasts (Fig. 6D) and that this effect was suppressed by pretreating cells with amlodipine or with the other aforementioned CCBs or calcineurin inhibitors.

We also found that calcineurin activity was increased by Fas stimulation and that this increase was significantly suppressed not only by the calcineurin inhibitors but also by amlodipine (Fig. 6E), which suggests that calcium influx through voltage-gated calcium channels and activation of the calcineurin pathway play important roles in mediating Fas-induced apoptosis among infarct tissue-derived myofibroblasts.

DISCUSSION

Effect of Amlodipine on Postinfarction Processes in Mice

Consistent with earlier reports (20, 23), we found that administering the L-type CCB amlodipine, beginning on the third day after the onset of MI, improved survival and cardiac structure and function at the chronic stage. Treatment with hydralazine, in contrast, had no beneficial effect, despite lowering blood pressure to the same degree as did amlodipine. Thus, amlodipine apparently affects postinfarction processes via a mechanism that is largely independent of its ability to lower blood pressure.

We found that amlodipine alters the geometry of the infarct scar without affecting its absolute area, i.e., the infarcted segment was thicker and had a smaller circumferential length in amlodipine-treated hearts during the chronic stage than in either saline- or hydralazine-treated hearts. This is noteworthy because wall stress is directly proportional to cavity diameter and inversely proportional to wall thickness (Laplace’s law) (31) and because wall stress and LV remodeling (dilatation) have a vicious relationship and accelerate one another. It is thus conceivable that the observed change in infarct geometry would have a highly beneficial effect on cardiac function.

Effect of Amlodipine on Nonmyocyte Dynamics in the Infarcted Area

Our findings also demonstrate that infarct scar tissue is qualitatively altered in mice administered amlodipine. We observed greater numbers of cells, including myofibroblasts...
and vascular cells, within the infarct scar in amlodipine-treated hearts. These cells are normally destined to disappear via apoptosis over the natural course of healing (25), but we found that apoptosis was significantly suppressed in amlodipine-treated hearts during the subacute stage (10 days post-MI), which has two important implications.

First, the granulation tissue cells that escape apoptotic cell death during the subacute stage may contribute to the cell population seen within the scar tissue during the chronic stage and preserve the infarct wall thickness. Myofibroblasts, in particular, are well known to play an important role in wound contraction during the healing process (4) and could mediate contractile reduction of the infarct segment length, thereby increasing infarct wall thickness. That, in turn, would alter infarct tissue geometry, reducing wall stress and mitigating LV dilatation and dysfunction. Although myofibroblasts, which can also produce collagen (4), were more abundantly preserved in the scar tissue of the amlodipine group, no excessive fibrosis was observed there. Since collagen production is generally stimulated by mechanical stretch or fibrogenic cytokines in (myo)fibroblasts, this finding might imply that myofibroblasts were not so activated to produce collagen in amlodipine-treated hearts with attenuated dysfunction. This idea may be supported by evidence that calcium channel blockade attenuates cardiac fibrosis, which develops in postinfarction, hypertensive, or cardiomyopathic hearts or that induced by angiotensin II or aldosterone (16, 20, 23, 27, 28). However, further studies are needed to elucidate whether amlodipine indeed reduces collagen production in (myo)fibroblasts and, if so, how the mechanisms are related. Vascular endothelial cells also escaped apoptotic death during the granulation tissue phase in hearts treated with amlodipine; however, the functional significance of these cells during the chronic stage of MI is unknown. It may be that by supplying blood these vessels help sustain the cellular components within the infarcted area (24). Macrophages, on the other hand, continued to die. We suggest that macrophages may have a higher sensitivity to apoptotic stimuli than other cells, as inflammatory cells generally exhibit very active proapoptotic interactions mediated via death ligands and receptors (10).

The second implication is that calcium influx through L-type calcium channels and the resultant calcium-evoked signaling exert a proapoptotic effect on granulation tissue cells in infarcted hearts and that, by inhibiting the influx of calcium, amlodipine exerts an antiapoptotic effect.

**Molecular Mechanism Underlying Amlodipine’s Inhibitory Effect on Granulation Tissue Cell Apoptosis**

Elevation of cytosolic calcium reportedly induces apoptosis in some cell types through the activation of calcineurin (3). In the present study, we found that calcineurin activity was elevated in infarcted hearts and that granulation tissue cell apoptosis within the infarcted area was calcineurin dependent, i.e., this apoptosis was suppressed by both CCBs and calcineurin inhibitors. Moreover, our in vitro analysis showed that calcineurin activity in granulation tissue-derived myofibroblasts was increased by Fas stimulation, and this effect was significantly suppressed by amlodipine as well as by calcineurin inhibitors. Calcineurin is able to dephosphorylate Bad, which, in turn, promotes cell death, at least in part through heterodimerization with the antiapoptotic proteins Bcl-2 and Bcl-xL on the mitochondrial membrane. Our finding that the level of Bad phosphorylation is significantly lower in infarcted hearts than sham-operated hearts and that Bad phosphorylation is significantly restored by treatment with amlodipine suggests the calcineurin-Bad pathway plays a crucial role in granulation tissue cell apoptosis and that amlodipine likely suppresses apoptosis by interfering with that pathway.

We confirmed that the expressions of Fas and Fas ligand are upregulated in granulation tissue 10 days post-MI. However, the mechanism by which amlodipine inhibits apoptosis does not appear to involve either the classical and alternate pathways downstream of Fas, as amlodipine did not affect FADD expression and caspase-8 activation (classic pathway) or Daxx expression and JNK activation (alternate pathway). Nevertheless, our in vitro study showed that amlodipine did inhibit Fas-induced apoptosis among granulation tissue-derived myofibroblasts. Collectively, these findings suggest that calcium influx through the L-type calcium channel is, at least in part, necessary for the execution of Fas-induced apoptosis. Further investigation will be required to clarify the precise relationship between Fas signaling and calcium-dependent signaling in apoptosis.

Fig. 5. A: Western blot analysis of Bcl-2, Bcl-xL, Bax, Bad, and the phosphorylated form of Bad (P-Bad) in hearts at 10 days after surgery. B: calcineurin activity in hearts 10 days after surgery. Hearts were collected from sham-operated, saline-treated, hydralazine-treated, and amlodipine-treated mice. *P < 0.05 vs. the sham group; #P < 0.05 vs. the saline group; +P < 0.05 vs. the hydralazine group.
Study Limitations

Granulation tissue consists of many cell types, although in the present study we focused only representative cell types such as myofibroblasts, endothelial cells, and macrophages. It would have been desirable to clarify cell dynamics with the molecular signals in every cell type of granulation tissue, although we could not do so in the present study because of technical difficulty.

In earlier studies (5, 8), we have proposed that the inhibition of granulation tissue apoptosis mitigates post-MI cardiac dysfunction, and amlodipine was found to do so here. However, other effects of amlodipine, not only on the infarcted area but also on the noninfarcted area, have been reported, i.e., phenotypic changes in sarcomeric proteins and anti-inflammatory, antioxidant, and antifibrotic effects (16, 20, 23, 27, 28). We actually confirmed the antifibrotic effect of amlodipine in the noninfarcted area in the present study. Thus, the relative importance of the antiapoptotic effect, compared with amlodipine’s other effects, remains to be determined in terms of the benefits for postinfarction hearts.

Conclusions

Amlodipine inhibits Fas-mediated granulation tissue cell apoptosis in infarcted hearts, possibly by attenuating the activities of calcineurin and Bad. This finding may provide new insight into the mechanism by which CCBs attenuate postinfarction cardiac remodeling and dysfunction.

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