Inhibition of Fas-associated apoptosis in granulation tissue cells accompanies attenuation of postinfarction left ventricular remodeling by olmesartan

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Kanamori H, Takemura G, Li Y, Okada H, Maruyama R, Aoyama T, Miyata S, Esaki M, Ogino A, Nakagawa M, Ushikoshi H, Kawasaki M, Minatoguchi S, Fujiwara H. Inhibition of Fas-associated apoptosis in granulation tissue cells accompanies attenuation of postinfarction left ventricular remodeling by olmesartan. Am J Physiol Heart Circ Physiol 292: H2184–H2194, 2007. First published January 5, 2007; doi:10.1152/ajpheart.01235.2006.—Blockade of angiotensin II type 1 receptor (AT1) signaling attenuates heart failure following myocardial infarction (MI), perhaps through reduction of fibrosis in the noninfarcted myocardium. However, its specific effect on the infarct tissue itself has not been fully clarified, which we examined in the present study. After MI induction in mice, treatment with the AT1 blocker olmesartan, beginning on the 3rd day post-MI, significantly improved survival (94%) 4 wk post-MI, compared with saline (53%) and hydralazine (73%). Olmesartan-treated mice also showed significant attenuation of left ventricular dilatation and dysfunction, as well as significantly greater infarct wall thickness, although the absolute size of the infarct scar was unchanged. In addition, significantly greater numbers of nonmyocytes (mainly vascular cells and myofibroblasts) were present within the infarct scar in olmesartan-treated hearts. Ten days post-MI, apoptosis among granulation tissue cells was significantly suppressed in the olmesartan-treated hearts, where expression of Fas, Bax, procaspase-3, and Daxx and activation of caspase-3, c-Jun NH2-terminal kinase, and c-Jun were all significantly attenuated. By contrast, expression of Fas ligand, Bel-2, and Fas-associated death domain and activation of caspase-8 were unaffected, suggesting olmesartan exerts a negative regulatory effect on the alternate pathway downstream of Fas receptor. In vitro, olmesartan dose-dependently inhibited Fas-mediated apoptosis in granulation tissue-derived myofibroblasts. The present study proposes this antiapoptotic effect as another important mechanism for an AT1 blocker in improving post-MI ventricular remodeling, as well as its antifibrotic effect, and also suggests a significant link between renin-angiotensin and Fas/Fas ligand systems in postinfarction hearts.

angiotensin; apoptosis; heart failure; myocardial infarction

LARGE MYOCARDIAL INFARCTS (MIs) cause severe, chronic heart failure with unfavorable remodeling of the left ventricle (LV) that is characterized by LV dilatation and diminished cardiac performance (22). Although the magnitude of the acute MI, which can be determined within several hours of an attack (23), is the most critical determinant of subsequent heart failure, many other factors, including late death or hypertrophy of cardiomyocytes, fibrosis, and expression of various cytokines, also are associated with disease progression (4, 18, 27, 34). This is because infarct 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 (29, 33, 35). Most of the cell components that infiltrate and proliferate within the infarct, including inflammatory and granulation tissue cells, disappear via apoptosis during the subacute and acute stages (5, 30). We found that granulation tissue cell apoptosis is Fas and caspase dependent, and that its inhibition during the subacute stage of MI altered infarct tissue dynamics, resulting in mitigation of adverse LV remodeling and dysfunction at the chronic stage (8, 16).

Evidence obtained from experiments using angiotensin converting enzyme inhibitors (ACEIs), angiotensin II type 1 receptor (AT1) blockers (ARBs), and genetically engineered animals lacking angiotensin II type 1A receptor (AT1A) has shown that AT1 signaling is a critical mediator of the progression of post-MI LV remodeling and heart failure (7, 26, 28). In that regard, myocardial fibrosis within noninfarcted tissue, which increases myocardial stiffness, is known to be diminished by AT1 signaling blockade, perhaps resulting in improved cardiac performance. It remains unclear, however, whether diminished fibrosis within the noninfarcted tissue could, by itself, exert a positive effect. In addition, little is known about the effects of AT1 signaling on the infarct tissue. Using AT1A knockout mice, we have very recently shown that AT1A signaling significantly affected the dynamics of infarct tissue, thereby influencing the postinfection disease process (17). Namely, in AT1A knockout hearts, granulation tissue cell preservation through increased proliferation and decreased apoptosis may have contributed to an abundant cell population to alter the infarct tissue structure, thereby reducing wall stress and attenuating LV dilatation and dysfunction at the chronic stage. We thus concluded that altered structural dynamics of infarct scar as well as increasing myocardial fibrosis may be responsible for the deleterious effects of AT1A signaling following MI (17). In that study, we, furthermore, suggested a possible involvement of Akt, which was activated by AT1A blockade, in increased proliferation of granulation tissue cells, whereas the molecular mechanisms of antiapoptosis were not well investigated (17). In the present study, to further verify this hypothesis, we first performed a morphological analysis of AT1-mediated alterations in post-MI hearts by inhibiting AT1 signaling with the ARB olmesartan. Then we sought to elucidate the pathophysiological and

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Molecular mechanisms underlying the observed effects, anti-apoptotic effect in particular.

MATERIALS AND METHODS

In Vivo Animal Experiments

This study was approved by our Institutional Animal Research Committee. MI was induced in 10-wk-old male C57BL/6J mice (Chubu Kagaku) by ligating the left coronary artery \( (n/H_11005) \), as previously described (16). In sham-operated mice \( (n/H_11005) \), the suture was passed but not tied.

On the 3rd day after surgery, 76 mice in the MI group remained alive (survival rate, 83%), while all of the mice in the sham-operated group survived. The 76 MI-bearing mice were randomly assigned to three groups: a saline group, which served as the untreated control group; a hydralazine group \( (15 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}; \text{Sigma}) \), which served as the hypotension control group; and an olmesartan group \( (10 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}; \text{a gift from Sankyo Pharmaceutical, Tokyo, Japan}) \). Before the treatment protocols were initiated, all three groups showed similar cardiac function on echocardiography. The reagents were administered using subcutaneously embedded osmotic minipumps (ALZET), beginning on the 3rd day after surgery and continued until death. Animals were killed 10 days or 4 wk after surgery.

Cell Culture and Treatment

Ten days after MI was induced in the mice, cardiac myofibroblasts were collected from the infarcted areas of the hearts, as described previously, with some modification (13). Briefly, the hearts were resected, and the infarcted areas were removed. The tissue was then minced and incubated with collagenase type II (Worthington) in Krebs-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 \( \alpha \)-smooth muscle actin (SMA) positive. These cells were used for experimentation at the second passage. Medium was replaced with serum-free DMEM for 24 h, and a mixture of agonistic anti-Fas antibody \( (1 \mu\text{g}/\text{ml}, \text{Pharmingen}) \) and actinomycin D \( (0.05 \mu\text{g/ml}, \text{Sigma}) \) was applied to the cells for 24 h to induce apoptosis (20). In some experiments, the cells were simultaneously treated with angiotensin II \( (\text{Calbiochem}) \) or pretreated with olmesartan, captopril \( (\text{an ACEI}; \text{ICN Biomedicals}), \text{losartan (an ARB}; \text{LKT Laboratories}), \text{SOD (Sigma), catalase (Sigma), or defereroxamine (Sigma) before the addition of the anti-Fas antibody and actinomycin D.}

Physiological Studies

Blood pressure was measured before and at 2 and 4 wk after surgery using the tail cuff method (BP Monitor, Muromachi Kikai). Physiological studies (echocardiography and cardiac catheterization) were carried out as described previously with modifications (16). Animals were anesthetized with halothane (induction, 2%; maintenance, 0.5%) in a mixture of N2O and O2 \( (0.5 \text{ l/min each}) \) via a nasal mask. Echocardiograms were recorded using an echocardiographic system (Vevo770, Visualsonics) equipped with a 45-MHz imaging system.
transducer before treatment and at death. Following echocardiography, the right carotid artery was cannulated with a micromanometer-tipped catheter (SPR 671, Millar Instrument) that was advanced into the aorta and then into the LV for recording pressure and maximal and minimal change in pressure over time.

**Histology**

Once the physiological measurements were complete, all surviving mice were killed, and the hearts were removed and cut into two transverse slices through the middle of the infarct, between the atrioventricular groove and the apex. The basal specimens were fixed in 10% buffered formalin, embedded in paraffin, cut into 4-μm-thick sections, and stained with hematoxylin-eosin, Masson’s trichrome, and Sirius red F3BA (0.1% solution in saturated aqueous picric acid; Aldrich). Quantitative assessments, including cell size and cell number, were carried out in randomly chosen high-power fields (HPFs) in each section; morphometrical analyses of the areas of the infarct, fibrosis, and immunopositive cells were carried out using 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, the 4-μm-thick sections were incubated with a primary antibody against α-SMA (1A4, Sigma), endothelial cells (Flk-1, Santa Cruz), macrophages (Mac3, Biomedicals AG), or 8-hydroxy-2′-deoxyguanosine (8-OHdG; Japan Institute of The Control of Aging, Shizuoka, Japan), which is a modified DNA base product most commonly used to evaluate oxidative DNA damage (31). 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 number or area of the immu-

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**Fig. 2. Gross morphology, histology, and immunohistochemistry.**

**A:** transverse ventricular sections collected from U, H, and O mice 4 wk post-MI. The sections are stained with Masson’s trichrome. Note the smaller LV cavity, shorter infarct segment, and thicker infarct wall in the olmesartan-treated heart. Bars, 1 mm. **B:** infarct areas in hearts from U, H, or O mice stained with hematoxylin-eosin (HE), anti-α-smooth muscle actin (SMA), anti-Flk-1, or anti-Mac3 (CD45). Bars, 20 μm.
nonspecific cells, were made in 20 randomly chosen HPFs using a multipurpose color image processor.

In situ terminal dUTP nick end-labeling (TUNEL) assays were carried out with sections using an ApoTag Kit (Chemicon), according to the supplier’s instructions. Mouse mammary tissue served as a positive control.

For double-label immunofluorescence, TUNEL, and the active form of caspase-3, sections were respectively labeled using Fluorescein-FragEL (Oncogene) and a primary antibody against the active form of caspase-3 (Chemicon) that was subsequently labeled with an Alexa 488-conjugated secondary antibody (Molecular Probes). Sections were then counterstained with Hoechst 33342 and observed under a confocal microscope (LSM510, Zeiss).

**Western Blotting**

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. The membranes were then probed using primary antibodies against Fas, Fas ligand (both from BD Transduction Laboratories/BD Pharmingen), Fas-associated death domain (FADD), Daxx (both from Santa Cruz), caspase-8, caspase-3 (both from Cell Signaling), Bcl-2, Bax (both from Santa Cruz), extracellular signal-regulated kinase (ERK), the phosphorylated (activated) form of ERK (both from Cell Signaling), c-Jun NH2-terminal kinase (JNK), the phosphorylated form of JNK (both from Santa Cruz), p38 mitogen-activated protein kinase (MAPK) (p38), the phosphorylated form of p38 MAPK (both from Sigma), c-Jun, and the phosphorylated form of c-Jun (both from Cell Signaling), after which the blots were visualized using chemiluminescence (ECL, Amersham). α-Tubulin (analyzed using an antibody from Santa Cruz) or β-actin (antibody from Sigma) served as the loading control.

**Statistical Analysis**

Values are shown as means ± SD. Survival was analyzed using the Kaplan-Meier method with the log-rank Cox-Mantel method. The significance of differences between groups was evaluated using t-tests or one-way ANOVA with a post hoc Newman-Keuls multiple-comparisons test. Values of P < 0.05 were considered significant.

**RESULTS**

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

The survival rates among the 6 sham-operated, 15 saline-treated, 15 hydralazine-treated, and 18 olmesartan-treated mice were evaluated 4 wk after induction of MI. All sham-operated mice survived. The survival rate among the MI was significantly greater in the olmesartan group (94%) than in the saline group (54%), but there was no significant difference between the hydralazine (73%) and saline groups (Fig. 1A).

Systolic blood pressures did not significantly differ between mice in the hydralazine and olmesartan groups, both of which were lower than in the saline group (Fig. 1B). Echocardiography and cardiac catheterization carried out 4 wk post-MI revealed severe LV remodeling with marked enlargement of the LV cavity and signs of diminished cardiac function, i.e., reduced LV ejection fraction, reduced maximal and minimal change in pressure over time, and increased LV end-diastolic pressure (Fig. 1C). The heart rate during echocardiographic examination was similar between the groups. Each of these conditions was significantly attenuated in olmesartan-treated mice, but not in hydralazine-treated mice. It thus appears that olmesartan mitigates post-MI remodeling and improves cardiac function via a mechanism that is largely independent of its ability to lower blood pressure.

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

**Gross morphometry.** Heart weight-to-body weight ratios were significantly smaller in the olmesartan group (4.8 ± 0.82 mg/g) than the control groups (untreated, 5.9 ± 1.4 mg/g and hydralazine, 5.6 ± 0.54 mg/g). Whereas hearts from saline- and hydralazine-treated mice showed marked LV dilatation with a thin infarcted segment, those from olmesartan-treated mice showed substantially smaller LV cavities and thicker infarcted segments with shorter circumferential lengths (Fig. 2A and Table 1). 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 the three groups (Table 1).

**Infarcted area.** By 4 wk post-MI, the infarct area had been replaced by fibrous scar tissue in the saline- and hydralazine-treated mice (Fig. 2B). In olmesartan-treated mice, by contrast, not only collagen fibers but also abundant cellular components were present. The population of noncardiomyocyte interstitial cells within the infarct area was significantly greater, and thus the relative percentage of fibrotic tissue was significantly smaller in the olmesartan-treated mice (Fig. 2B and Table 1). The number of Flk-1-positive blood vessels present within the infarct area and the percent area of extravascular α-SMA-positive cells were greater in the olmesartan-treated mice than in the other two groups (Fig. 2B and Table 1). Few macrophages were observed in any of the groups, and their incidences were similar among the three groups.

**Noninfarcted area.** Sirius red staining showed there to be significantly less fibrosis in the noninfarcted LV walls of the olmesartan-treated mice than in those of saline- and hydralazine-treated mice (Table 1). In addition, the transverse diameters of the cardiomyocytes in the noninfarcted area were significantly greater in the saline and hydralazine groups than in the olmesartan group (Table 1), suggesting that compensatory hypertrophy of cardiomyocytes was less developed in the olmesartan group.

| Table 1. Effects of treating mice with saline, hydralazine, or olmesartan on the morphometry and histology of hearts bearing 4-wk-old MIs |
|-----------------|-----------------|-----------------|
| Saline          | Hydralazine     | Olmesartan      |
| Injured area    |                 |                 |
| MI area in LV, %| 23.2 ± 7.9      | 20.7 ± 5.7      | 23.5 ± 8.8      |
| MI thickness, ×10^3 μm | 2.28 ± 0.26    | 2.63 ± 0.61    | 3.56 ± 0.93 †   |
| MI segment length, mm | 8.29 ± 2.1    | 6.56 ± 1.7      | 4.71 ± 0.99 †   |
| MI segment length in LV, % | 53.2 ± 11.1   | 52.7 ± 10.1     | 41.7 ± 6.08 †   |
| Cell population, cells/mm² | 310 ± 49       | 392 ± 42*       | 470 ± 58* †     |
| Fibrosis, %     | 56 ± 3.6        | 54 ± 1.4        | 43 ± 3.3 †      |
| Flk-1⁺ vessels per HPF | 5.8 ± 1.3       | 14.4 ± 5.4 *  | 26.2 ± 2.7 †    |
| α-SMA⁺ cells, % | 2.61 ± 0.52     | 2.39 ± 0.60     | 4.09 ± 0.71 †   |
| Mouse ⁺ cells per HPF | 4.9 ± 1.0       | 6.0 ± 2.2       | 5.1 ± 1.4       |

Values are means ± SD; n, no. of mice. MI, myocardial infarction; LV, left ventricular; HPF, high-power field; SMA, smooth muscle actin. *P < 0.05 vs. the saline group; †P < 0.05 vs. the hydralazine group.
Granulation Tissue Cell Dynamics 10 Days Post-MI

As mentioned above, we found greater numbers of cells, especially α-SMA-positive and endothelial cells, within the infarct areas of the olmesartan-treated hearts during the chronic stage of MI (4 wk post-MI). Because we thought that the dynamics of the cells during the ongoing healing process might explain the difference in the composition of the infarct tissue 4 wk post-MI, we next evaluated the incidence of apoptosis among granulation tissue cells in saline- (n = 8), hydralazine- (n = 10), and olmesartan-treated (n = 10) hearts 10 days post-MI, the time when granulation tissue peaks in mice (subacute stage of MI). Apoptosis among granulation tissue cells was detectable in TUNEL assays in hearts from all three groups (Fig. 3A). In addition, Western analysis revealed the presence of the active forms of caspase-3 and caspase-8 in the infarcted hearts (Fig. 4), while double-label immunofluorescence assays for TUNEL and the active form of caspase-3 revealed the presence of both in some of the granulation tissue cells (Fig. 3B). Notably, we found that the incidence of TUNEL-positive cells was significantly smaller in the olmesartan-treated hearts than in the other two groups (Fig. 3A, graph). In addition, 8-OHdG positivity, an indicator of oxidative DNA damage (19), was significantly diminished in granulation tissue cells from olmesartan-treated hearts (Fig. 3C), suggesting a possible link between oxidative damage and apoptosis in these cells. By contrast, we detected virtually no TUNEL-positive cardiomyocytes in any of the groups.

Fig. 3. Apoptosis and oxidative damage in granulation tissue 10 days post-MI. A: photomicrographs showing terminal dUTP nick-end labeling (TUNEL)-positive cells and a graph comparing the incidences of TUNEL positivity in the U, H, and O groups. Bars, 20 μm. B: immunofluorescence images showing the distribution of TUNEL (green) and the active form of caspase-3 (red) in granulation tissue cells in a saline-treated heart. Nuclei were stained with Hoechst (blue). Arrow indicates a double-positive cell. Bar, 20 μm. C: photomicrographs showing 8-hydroxy-2′-deoxyguanosine (8-OHdG)-positive cells and a graph comparing the incidences of 8-OHdG positivity in the U, H, and O groups. Bars, 20 μm. *P < 0.05 vs. the U group; #P < 0.05 vs. the H group.
Proliferating activity examined by Ki-67 immunohistochemistry revealed that the immunopositive cells were significantly more frequent in granulation tissue of the olmesartan-treated group (5.5 ± 1.9%) than in that of the control group (2.1 ± 1.0%, P < 0.05), supporting our previous finding using AT1A knockout mice (17).

**Apoptosis-Related Molecular Signaling in Granulation Tissue Cells**

We previously found that post-MI granulation tissue cell apoptosis is, at least in part, Fas dependent (16). Bearing that in mind, we next used Western analysis to investigate apoptosis-related molecular signaling, especially in the Fas pathway, in granulation tissue collected 10 days post-MI. Figure 4 shows the representative blots. We found that expression of both Fas and Fas ligand was significantly upregulated in granulation tissue from mice in all three groups, although the level of Fas expression was significantly lower in olmesartan-treated hearts than in saline- or hydralazine-treated hearts. The proapoptotic protein Bax also was significantly upregulated in all three groups, but again the degree of upregulation was significantly lower in olmesartan-treated hearts. Expression of Bcl-2, slightly upregulated in hearts with MI, was not affected by the hydralazine or olmesartan treatment. Interestingly, although expression of procaspase-3 was upregulated in granulation tissue from all of the hearts 10 days post-MI, its levels were highest in the saline group and higher in the hydralazine group than in the olmesartan group. Expression of the active forms of caspase-3 (17 and 19 kDa) was significantly lower in the olmesartan group than in the others, whereas expression of the active form of caspase-8 (45 kDa) was similar among all three groups 10 days post-MI.

Two distinct signal transduction pathways have been identified downstream of Fas receptor: the classic and the alternate pathways (9, 36). In the classic pathway, FADD is recruited via the death domain of Fas receptor, whereas the Fas binding protein Daxx is involved in the alternate pathway. We observed a significant upregulation of Daxx, but not of FADD, in granulation tissue 10 days post-MI, although the level of Daxx expression was lower in the olmesartan group than in the saline or hydralazine group (Fig. 5). We then assessed the expression and activation of several MAPKs known to serve as components of the alternate pathway and found that expression of ERK and JNK, but not p38, was enhanced in all three groups 10 days post-MI (Fig. 5). When we then assessed levels of activated (phosphorylated) MAPKs, we found that phosphorylated-ERK levels were significantly higher in the hydralazine and olmesartan groups than in the saline group, that phosphorylated-p38 levels were similar in all three groups, and that phosphorylated-JNK levels were significantly attenuated in the olmesartan group, as were phosphorylated-c-Jun levels (Fig. 5).

**Effect of Olmesartan on Fas-induced Apoptosis Among Cultured Myofibroblasts**

Incubating cultured myofibroblasts derived from granulation tissue with agonistic anti-Fas antibody plus actinomycin D for...
24 h induced apoptosis in 23 ± 5.9% of the cells. Pretreatment with olmesartan suppressed this apoptosis in a dose-dependent manner, and this effect was accompanied by inhibition of caspase-3 and JNK activation (Fig. 6, A and B). Pretreatment with captopril (an ACEI) or losartan (an ARB) similarly inhibited Fas-induced apoptosis (Fig. 6C).

Next, to examine what degree Fas and angiotensin II are required to induce apoptosis in myofibroblast, we treated cultured myofibroblasts with exogenous angiotensin II. Angiotensin II was previously reported to be produced and secreted from the cultured myofibroblasts that were derived from MI tissue in a serum-free condition (13). When angiotensin II was added at the concentrations of 5 to 5 × 10^5 pg/ml simultaneously with Fas stimulation, angiotensin II dose-dependently increased myofibroblast apoptosis, although angiotensin II alone at the examined doses failed in induction of significant apoptosis (Fig. 6D). This suggests that Fas stimulation is inevitable for induction of myofibroblast apoptosis, while angiotensin II augments it.

Finally, because a possible link between oxidative DNA damage and apoptosis was suggested by our in vivo experiments, we also pretreated myofibroblast cultures with antioxidants SOD and catalase and an iron chelator deferoxamine before induction of apoptosis. We found that all of these reagents effectively and dose-dependently suppressed Fas-induced apoptosis among the cultured myofibroblasts (Fig. 6E).

**DISCUSSION**

**Effect of Olmesartan on the MI Scar**

Myocardial fibrosis leads to both systolic and diastolic dysfunction in the heart (3). Moreover, it is well known that blockade of AT1 signaling using an ARB favorably affects post-MI heart function by suppressing excessive fibrosis in...
noninfarcted heart tissue (14–16). The specific effects of AT1 blockade on the infarct scar have not been well characterized, however. We have demonstrated here that AT1 blockade 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 olmesartan-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) (37), 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. Our laboratory actually observed in our previous study that, in mice lacking functioning Fas (lpr strain) and in those lacking Fas ligand (gld strain), apoptotic rate of granulation tissue cells was significantly fewer compared with that of genetically controlled mice, and post-MI ventricular remodeling and dysfunction were greatly attenuated (16). That said, the relative contributions made by this mechanism and antifibrotic effect (which numerous studies have demonstrated) to improved LV structure and function remain

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**Fig. 6.** In vitro experiments using granulation tissue-derived myofibroblasts. A: immunofluorescence images showing the distribution of TUNEL (green) and α-SMA (red) in the cultured myofibroblasts. FA, treatment with agonistic anti-Fas antibody (Ab) + actinomycin D (Act D). Bars, 10 μm. The graph shows the dose-dependent effect of olmesartan on Fas-mediated apoptosis among cultured myofibroblasts. B: Western blots with the densitometric analyses illustrating the dose-dependent effect of olmesartan on levels of the active form of caspase-3 and p-JNK in cultured myofibroblasts. C: effect of captopril and losartan on Fas-mediated apoptosis among cultured myofibroblasts. D: effect of exogenous angiotensin II (ANG II) on Fas-mediated apoptosis among cultured myofibroblasts. E: effect of SOD, catalase (CAT), and deferroxamine (DFX) on Fas-mediated apoptosis among cultured myofibroblasts. *P < 0.05 vs. the FA-treated group without pretreatment. FA, agonistic anti-Fas Ab (1 μg/ml) plus Act D (0.05 μg/ml).
Effect of Olmesartan on Granulation Tissue Cell Dynamics

Our findings also demonstrate that infarct scar tissue is qualitatively altered by treatment with olmesartan. We observed a greater numbers of cells, including abundant myofibroblasts and vascular cells, within the infarct scar in olmesartan-treated hearts. These cells are normally destined to disappear via apoptosis over the natural course of healing (5, 29), but we found that apoptosis was significantly suppressed in olmesartan-treated hearts during the subacute stage (10 days post-MI). This finding has two important implications. First, the granulation tissue cells that escaped from apoptotic death during the subacute stage might contribute to the increased cell population seen within the scar tissue at 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 (6) and could mediate contractile reduction of the infarct segment, thereby increasing infarct wall thickness. That, in turn, would alter infarct tissue geometry, reducing wall stress and mitigating LV dilatation and dysfunction. Vascular endothelial cells also escaped apoptotic death during the granulation tissue phase in hearts treated with olmesartan; however, the function 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 (29). Macrophages, on the other hand, continued to die. Speculatively, macrophages may have a higher sensitivity to apoptotic stimuli compared with the other preserved cells, because inflammatory cells generally show very active proapoptotic interactions through death ligands and receptors (19).

The second implication is that AT1 signaling exerts a proapoptotic effect on granulation tissue cells in infarcted hearts.

Molecular Mechanisms for Inhibition of Granulation Tissue Cell Apoptosis by Olmesartan

The present study confirmed that Fas and Fas ligand are overexpressed in granulation tissue during the subacute stage of MI (16). Two distinct signal transduction pathways, the classic and the alternate pathways, have been identified downstream of Fas receptor (9, 36), and a significant upregulation of Daxx (alternate pathway), but not of FADD (classical pathway), was observed in the hearts 10 days post-MI. ERK and JNK, two MAPKs situated downstream of Daxx, also were activated in the infarcted hearts. This strongly suggests the alternate pathway, not the classic pathway, plays the predominant role during induction of apoptosis in granulation tissue. Consistent with this notion is the fact that inhibition of apoptosis by olmesartan was accompanied by reductions in Daxx expression and JNK activity, but not in FADD expression or caspase-8 activity. Furthermore, levels of activated c-Jun, one of the components of the transcription factor activator protein-1, were enhanced. Activator protein-1 is reportedly activated by Fas stimulation in several cell lines (12, 21) and is known to be a potential enhancer of fas and bax genes (11, 24).

Indeed, we observed upregulation of both Fas and Bax, as well as procaspase-3, in post-MI granulation tissue.

We also found that olmesartan significantly suppressed granulation tissue cell apoptosis through effects on the expression of Fas itself, as well as on the expression and activation of various signaling molecules situated downstream of Fas. Moreover, our in vitro experiment using granulation tissue-derived myofibroblasts revealed olmesartan exerts a dose-dependent inhibitory effect on Fas-mediated apoptosis, as did captopril, an ACEI, and losartan, another ARB. These findings highlight an apparent linkage between the Fas/Fas ligand system and renin-angiotensin system in MI. To our knowledge, this is the first report of such a linkage. In addition, our immunohistochemical analysis of 8-OHdG levels revealed the presence of oxidative damage in granulation tissue cells in post-MI hearts, and this damage was significantly attenuated by olmesartan. We also found that the antioxidants significantly suppressed Fas-mediated apoptosis in granulation tissue-derived myofibroblasts. It is known that, by activating NADPH oxidase, AT1 signaling generates oxidative stress, which is an important inducer of apoptosis in a variety of cell types within the

Fig. 7. Proposed scheme showing a possible link in signal transduction between the Fas/Fas ligand system and the renin-angiotensin system (RAS) in post-MI granulation tissue cells. ANG II type 1 receptor (AT1) blocker (ARB) may exert an effect on the alternate pathway downstream of Fas receptor through inhibition of AT1-mediated ROS production. ASK-1, apoptosis signal-regulating kinase.

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cardiovascular system (1, 14, 15, 32). Oxidative stress exerts its apoptotic effect by stimulating apoptosis signal-regulating kinase 1, a MAPK kinase kinase, which in turn promotes apoptosis through activation of JNK and/or p38 (9). Apoptosis signal-regulating kinase 1 also is a component of the alternate pathway downstream of Fas. Collectively then, our findings suggest that oxidative stress represents the link between the Fas/Fas ligand system and the renin-angiotensin system in infarcted hearts (Fig. 7). Interestingly, AT1 signaling often stimulates cell proliferation by inducing expression of various progrowth cytokines and chemokines (2, 25), although the role of AT1 during angiogenesis within infarcted tissue is controversial (10). In any case, the net effect of the molecular signaling transduced via AT1 within infarct tissue appears to be a reduction in the cell population within granulation tissue.

Study Limitations

Although we have proposed that preservation of cells through escaping from apoptotic death in the infarcted area might at least, in part, contribute to attenuation of post-MI cardiac dysfunction in the present and previous studies (8, 16), this hypothesis remains to be established. However, this issue may hold true for the antiapoptosis hypothesis, because the difference in degree of reduction in myocardial fibrosis is not so great between the wild-type mice (~3%) and AT1A knockout mice (~1%), making its effectiveness suspicious (7). The relative importance for the benefits between the effects of AT1 blockade, i.e., this antiapoptosis and antifibrosis previously reported, remains to be determined.

Among the apoptosis-relating signals examined, expression of Fas, Daxx, and Bax and activation of JNK, Jun, and signal-regulating kinase 1 also is a component of the alternate Fas signaling pathway. Apoptosis by suppressing the alternate Fas signaling pathway, other factors. Might be affected, not only by AT1 blockade, but also by the other factors.

In conclusion, olmesartan inhibits granulation tissue cell apoptosis by suppressing the alternate Fas signaling pathway, thereby altering post-MI infarct tissue dynamics. This finding suggests that a link between renin-angiotensin and Fas-Fas ligand systems and may provide new insight into the mechanism by which inhibition of AT1 signaling improves LV structure and function in the post-MI heart.

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