Anakinra, a Recombinant Human Interleukin-1 Receptor Antagonist, Inhibits Apoptosis in Experimental Acute Myocardial Infarction

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Background—Experimental interleukin-1 receptor antagonist gene overexpression has shown that interleukin-1 receptor antagonist is cardioprotective during global cardiac ischemia. The aim of the present study was to test the impact of an exogenous recombinant human interleukin-1 receptor antagonist (anakinra) in experimental acute myocardial infarction.

Methods and Results—Two animal studies were conducted: one of immediate anakinra administration during ischemia in the mouse and one of delayed anakinra administration 24 hours after ischemia in the rat. Seventy-eight Institute of Cancer Research mice and 20 Wistar rats underwent surgical coronary artery ligation (or sham operation) and were treated with either anakinra 1 mg/kg or NaCl 0.9% (saline). Treatment was administered during surgery and then daily for 6 doses in the mice and starting on day 2 daily for 5 doses in the rats. Twenty-eight mice underwent infarct size assessment 24 hours after surgery, 6 saline-treated mice and 22 mice treated with increasing doses of anakinra (1 mg/kg [n=6], 10 mg/kg [n=6], and 100 mg/kg [n=10]); 6 mice were euthanized at 7 days for protein expression analysis. The remaining animals underwent transthoracic echocardiography before surgery and 7 days later just before death. Cardiomyocyte apoptosis was measured in the peri-infarct regions. The antiapoptotic effect of anakinra was tested in a primary rat cardiomyocyte culture during simulated ischemia and in vitro on caspase-1 and -9 activities. At 7 days, 15 of the 16 mice (94%) treated with anakinra were alive versus 11 of the 20 mice (55%) treated with saline ($P=0.013$). No differences in infarct size at 24 hours compared with saline were observed with the 1- and 10-mg/kg doses, whereas a 13% reduction in infarct size was found with the 100-mg/kg dose ($P=0.015$). Treatment with anakinra was associated with a significant reduction in cardiomyocyte apoptosis in both the immediate and delayed treatment groups ($3.1\pm0.2\%$ versus $0.5\pm0.3\%$ [$P<0.001$] and $4.2\pm0.4\%$ versus $1.1\pm0.2\%$ [$P<0.001$], respectively). Compared with saline-treated animals, anakinra-treated mice and rats showed signs of more favorable ventricular remodeling. In vitro, anakinra significantly prevented apoptosis induced by simulated ischemia and inhibited caspase-1 and -9 activities.

Conclusions—Administration of anakinra within 24 hours of acute myocardial infarction significantly ameliorates the remodeling process by inhibiting cardiomyocyte apoptosis in 2 different experimental animal models of AMI. This may open the door for using anakinra to prevent postischemic cardiac remodeling and heart failure. **(*Circulation*. 2008;117: 2670-2683.)**

Key Words: apoptosis ■ cytokine ■ heart failure ■ ischemia ■ pharmacology ■ remodeling

Acute myocardial infarction (AMI) is a major cause of morbidity and mortality worldwide. AMI is caused by sudden onset of myocardial ischemia and ensuing necrosis. AMI survivors remain at high risk of death in the years after the index event. Because treatment for AMI has significantly improved in the past years, more patients are surviving AMI. The most common complication of AMI is the occurrence of left ventricular dysfunction and heart failure. The initial

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ischemic damage to the myocardium activates a cascade of events that eventually lead to adverse cardiac remodeling and heart failure with ensuing excesses in morbidity and mortality.[1] The remodeling process occurring in the ischemic and nonischemic myocardium is mediated by upregulation and downregulation of different pathways that trigger cardiomyocyte hypertrophy and apoptosis in a delicate balance between death and survival.[1,2] Interleukin-1 (IL-1) receptor antagonist (IL-1Ra), a member of the IL-1 family, is a naturally occurring antiinflammatory protein that behaves as an acute-phase reactant.[3,4] Like other acute-phase reactants, IL-1Ra levels increase during AMI, and its levels correlate with prognosis.[5,6] The role of increasing IL-1Ra during AMI is unclear, and speculations have been made that its role ranges from merely a marker of damage to a modulator of the inflammatory response to a potential cytoprotective agent.[5,8] Recently, forced expression of IL-1Ra in an animal model has been shown to be cardioprotective in terms of reduced infarct size and apoptosis.[8]

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In the present study, we investigated the effects of anakinra, an exogenous recombinant human IL-1Ra, in 2 experimental models using left coronary artery surgical ligation in rodents: a study of immediate anakinra administration in the mouse model to assess its effects on apoptosis, infarct size, and remodeling and a study of delayed (24 hours) administration of anakinra to assess its effects on apoptosis independently of potential infarct-sparing effects and on cardiac remodeling in the rat model (to assess also for potential interspecies differences). Additionally, the antiapoptotic effect of anakinra was tested in vitro.

Methods

Surgical Procedures

All animals were supplied by Harlan Sprague Dawley (Indianapolis, Ind). All animal experiments were conducted under the guidance of the guidelines on the humane use and care of laboratory animals for biomedical research published by National Institutes of Health (No. 85-23, revised 1996). Adult male outbred Institute of Cancer Research mice (age, 10 weeks; weight, 26 to 38 g) and adult Wistar rats (age, 10 weeks; weight, 350 to 500 g) underwent coronary ligation. The surgical procedures were performed on day 1 by 2 skilled operators (F.N.S. and S.S.) as previously described.[9,10] The animals under anesthesia (pentobarbital 50 to 70 mg/kg) were intubated and placed on right decubitus and then underwent surgical opening of the chest and ligation of the proximal left coronary artery. Eight mice and 8 rats underwent a sham operation that included every step except coronary ligation; half were treated with daily anakinra (1 mg/kg) injections, and the remaining half were treated with saline injections. The Institutional Animal Care and Use Committee of Virginia Commonwealth University approved the study. Ten mice and 4 rats died in the immediate postoperative period and were not included in any of the analyses.

Treatment

Two substudies were conducted: immediate anakinra administration during ischemia in the mouse and delayed anakinra administration 24 hours after ischemia in the rat. In the mouse model, anakinra 1 mg/kg (corresponding to the recommended dose for the treatment of rheumatoid arthritis) was given intraperitoneally during surgery and then daily for 6 doses in 20 mice (16 with coronary ligation and 4 sham-operated). In the rat model, anakinra was given intraperitoneally on day 2 and then daily for 5 doses in 8 rats (4 with coronary ligation and 4 sham-operated). The remaining 24 mice (20 with coronary ligation and 4 sham-operated) and 12 rats (8 with coronary ligation and 4 sham-operated) received NaCl 0.9% (saline) injections. Two different rodent species were used to evaluate for potential interspecies differences. An additional 28 mice underwent infarct size assessment 24 hours after surgery: 6 saline-treated mice and 22 mice treated with increasing doses of anakinra (1 mg/kg [n=6], 10 mg/kg [n=6], and 100 mg/kg [n=10]) to assess the potential dose-dependent infarct-sparing effects of anakinra. Finally, 6 additional mice (3 treated with anakinra and 3 saline-treated) were euthanized 7 days after coronary ligation for evaluation of matrix metalloproteinase-9 (MMP-9) expression. A total of 78 mice and 20 rats were used in this study.

Infarct Size Assessment

Twenty-four hours after completion of the infarct protocol, the heart was quickly removed and mounted on a Langendorff apparatus. The coronary arteries were perfused with 0.9% NaCl containing 2.5 mmol/L $CaCl_2$. After the blood was washed out, ≈2 mL of 10% Evans blue dye was injected as a bolus into the aorta until most of the heart turned blue. The heart was perfused with saline to wash out the excess Evans blue. Finally, the heart was removed, frozen, and cut into 8 to 10 transverse slices from apex to base of equal thickness (≈1 mm). The slices were then incubated in a 10% triphenyl tetrazolium chloride solution in an isotonic phosphate buffer (pH 7.4) at room temperature for 30 minutes. The areas of infarcted tissue, the risk zone, and the whole left ventricle were determined by computer morphometry with BIOQUANT imaging software (BIOQUANT Image Analysis Corp, Nashville, Tenn). Infarct size was expressed as a percentage of the ischemic risk area, which was determined as a percentage of the left ventricle.

Echocardiography

Transthoracic echocardiography under light anesthesia (pentobarbital 30 to 50 mg/kg) was performed just before surgery and 7 days after surgery just before death. Doppler echocardiography was performed with the Vevo770 imaging system (VisualSonics Inc, Toronto, Ontario, Canada) and a 30-MHz probe in the mouse; an echocardiography system equipped with a 15-MHz phase-array transducer (Hewlett-Packard, Palo Alto, Calif) was used in the rat. The transducer was positioned on the left anterior side of the chest. The heart was first imaged in the 2-dimensional mode in the short-axis view of the left ventricle. The M-mode cursor was positioned perpendicular to the anterior and posterior wall to measure left ventricular (LV) end-diastolic and end-systolic diameters (LVEDD and LVESD, respectively). According to the American Society of Echocardiography recommendations,[11] M-mode images were then obtained at the level of the papillary muscles below the mitral valve tip. In the mouse, apical 4- and 5-chamber views also were obtained to measure transmitral flow, left ventricular outflow, and transaortic flow velocities. LV fractional shortening (FS) was calculated as follows: FS=(LVEDD−LVESD)/LVEDD×100. Ejection fraction was calculated with the Teichholz formula. Transmitral and left ventricle outflow tract pulsed Doppler flow spectra were obtained from the apical view. Measurement of the outflow tract flow was performed. Isovolumetric contraction (ICT) and relaxation (IRT) times and ejection time (ET) were measured. LV outflow tract (LVOT) flow velocity–time integral (AoVTI) also was measured. These data were used to calculate the Tei index (Tei index=ICT+IRT/ET)[12] and cardiac output ($CO=AoVTI\times\pi\times(LVOT\ diameter/2)^{2}\times$heart rate, where LVOT was measured as the cross-sectional area in the parasternal long-axis view). In humans, a higher Tei index is associated with both systolic and diastolic dysfunction and worse outcomes.[12] The allocation to different treatments was random, and the investigator performing and reading the echocardiogram was blinded to the treatment.

Pathology

On day 7, after echocardiography and under anesthesia, the abdominal aorta was cannulated with a polyethylene catheter, the thorax was opened, the aorta was filled with phosphate buffer (0.2 mol/L, pH 7.4) and heparin (100 IU), and the right atrium was cut to allow drainage. In rapid succession, the heart was arrested in diastole, and perfusion with phosphate-buffered formalin was started. In the rats only, 1 to 2 mL whole blood was obtained from the heart for cytokine plasma determination. The left ventricular chamber was filled with fixative for a 10-minute fixation. At the end of the procedure, transverse sections of the median third of the left ventricle were taken and stored in formalin for at least 48 hours. Apoptosis was defined by staining for terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling (TUNEL; DNA fragmentation, Oncor, Gaithersburg, Md). The detailed protocol was published elsewhere.[13] The peri-infarct area was defined as the zone bordering the infarct where viable myocardium was prevalent.[13] The apoptotic rate was expressed as the number of apoptotic cardiomyocytes on all cardiomyocytes per field. Costaining for TUNEL and muscle actin (prediluted anti-mouse α-sarcomeric actin antibody, Invitrogen, San Francisco, Calif) was performed to document cell type. We considered primarily apoptosis in cardiomyocytes but also measured apoptosis in the granulation tissue (actin-negative mononuclear cells) in the infarct areas.

The number of leukocyte in the myocardium was measured as the number of $CD45^+$ cells per 1 mm^2 (using an anti-mouse CD45 antibody, 1:100 dilution, Southern Biotech, Birmingham, Ala) and compared between anakinra- and saline-treated AMI mice. The apoptotic rate in the peri-infarct regions was calculated in 10 random fields, which cover almost the entire peri-infarct area. The investigators performing the cell count were unaware of the treatment allocation.

We measured myocardial fibrosis at 7 days after AMI in the mouse to address whether immediate anakinra use was associated with impaired infarct healing. Heart sections were stained with Masson's trichrome stains (Sigma-Aldrich, St Louis, Mo). Briefly, sections were mordanted in Bouin's solution overnight and washed in running tap water to remove the yellow. Sections were stained with Mayer's hematoxylin, Biebrich scarlet-acid fuchsin, working phosphotungstic/phosphomolybdic acid solution, and aniline blue for 5 minutes each and then placed in 1% acetic acid for 2 minutes. Sections were rinsed, dehydrated through alcohol, cleared in xylene, and mounted. Cytoplasm and muscle fibers appear red; collagen and nuclei appear blue. The areas of fibrosis and the whole left ventricle were determined by computer morphometry using a BIOQUANT imaging software, and the ratio was used to compute scar area expressed as a percentage of the left ventricle. The ratio of fibrosis to viable myocardium in the peri-infarct area (interstitial fibrosis) was determined by computer morphometry using a $\times 20$ enlargement and expressed as a percentage of surface area. Von Kossa staining (Diagnostic Biosystem, Pleasanton, Calif) was used to detect (pre-)necrotic myocardial calcium deposits.

Cytokine Levels

Whole blood was drawn in sodium citrate tubes in 9 rats (4 treated with anakinra and 5 treated with normal saline) at the time of death and immediately centrifuged a 1000*g* at 4°C for 10 minutes. The supernatant was collected and filtered through a 0.22-μm filter. Samples were thereafter stored at −20°C and subsequently analyzed. A multiplex cytokine bead array system (Bio-Plex Cytokine Assay, Bio-Rad, Hercules, Calif) was used according to the manufacturer's instructions to determine circulating levels of IL-1β, tumor necrosis factor-α, IL-6, and interferon-γ. The reaction mixture was read with the Bio-Plex protein array reader, and data were analyzed with the Bio-Plex Manager software program.

MMP Synthesis

MMP-9, or gelatinase B, was chosen as prototypal metalloproteinase upregulated after AMI and associated with adverse remodeling.[14,15] Total soluble protein was extracted from the infarct and peri-infarct myocardium 7 days after coronary ligation in 6 mice (3 treated with anakinra and 3 with saline) with a buffer of 20 mmol/L Tris, 150 mmol/L NaCl, 1 mmol/L EDTA, and 1 mmol/L EGTA. The homogenate was centrifuged at 14 000*g* for 10 minutes under 4°C, and the supernatant was recovered. Then, 50 μg protein from each sample was separated by 7.5% acrylamide gels, transferred to a nitrocellulose membrane, and then blocked with 5% nonfat dry milk in Tris-buffered saline Tween-20 (10 mmol/L Tris-HCl, pH 7.4, 100 mmol/L NaCl, and 0.1% Tween 20) for 1 hour. The membrane was then incubated with goat polyclonal primary antibody at a dilution of 1:1000 for MMP-9 (Santa Cruz Biotechnology, Santa Cruz, Calif) for 16 hours at 4°C before being washed and incubated with anti-rabbit horseradish peroxidase–conjugated secondary antibody (1:2000; Amersham Biosciences, Inc, Piscataway, NJ) for 1 hour. The blots were developed with a chemiluminescent system. Two bands were apparent, corresponding to the pro–MMP-9 and active MMP-9. The active band (105 kDa) of MMP-9 only was selected for measurement. The optical density for each band was scanned and quantified with densitometry and expressed as the ratio of active MMP-9 to β-actin.

Antiapoptotic Effects of Anakinra In Vitro

The ventricular cardiomyocytes were isolated from the rat with an enzymatic technique modified as previously described.[16] Briefly, the rat was anesthetized with pentobarbital sodium (100 mg/kg IP), and the heart was quickly removed from the chest. Within 3 minutes, the aortic opening was cannulated onto a Langendorff perfusion system, and the heart was retrogradely perfused. The enzymatic digestion was begun by adding collagenase type II (0.5 mg/mL each; Worthington Biochemical Corp, Lakewood, NJ) and protease type XIV (0.02 mg/mL) to the perfusion buffer and continued for ≈15 minutes. Then, 50 μmol/L Ca_2^+ was added to the enzyme solution to perfuse the heart for another 10 to 15 minutes. The digested ventricular tissue was cut into chunks and gently aspirated with a transfer pipette to facilitate the cell dissociation. The cell pellet was resuspended for a 3-step Ca_2^+ restoration procedure (ie, 125, 250, and 500 μmol/L Ca_2^+). The freshly isolated cardiomyocytes were then suspended in minimal essential medium (catalogue number M1018, pH 7.35 to 7.45, Sigma). The cells were then plated onto 35-mm cell culture dishes that were precoated with 20 μg/mL mouse laminin in phosphate-buffered saline with 1% penicillin-streptomycin for 1 hour. The cardiomyocytes were cultured in the presence of 5% CO_2 for 1 hour in a humidified incubator at 37°C, which allowed cardiomyocytes to attach to the dish surface before the experimental protocol. Cardiomyocytes were then subjected to simulated ischemia for 40 minutes by replacing the cell medium with an "ischemia buffer" that contained 118 mmol/L NaCl, 24 mmol/L $NaHCO_3$, 1.0 mmol/L NaH_2PO_4, 2.5 mmol/L $CaCl_2$–$2H_2O$, 1.2 mmol/L $MgCl_2$, 20 mmol/L sodium lactate, 16 mmol/L KCl, and 10 mmol/L 2-deoxyglucose (pH adjusted to 6.2). In addition, the cells were incubated under hypoxic conditions at 37°C during the entire simulated ischemia period by adjusting the tri-gas incubator to 1% to 2% O_2 and 5% CO_2. After 40 minutes, reperfusion was simulated by replacing the ischemic buffer with normal medium under normoxic conditions. At the same time, anakinra was added to the plate at increasing ($\times 100$) concentrations starting with a concentration of 25×10^{-16}g/mL up to a concentration of 25×10^{-6}g/mL (n=3 per group). An equivalent volume of normal medium was added to 3 plates to serve as controls. Plates were incubated for 18 hours. Cardiomyocyte apoptosis was analyzed by TUNEL staining with a kit purchased from BD Biosciences (San Jose, Calif) that detects nuclear DNA fragmentation via a fluorescence assay. In brief, after 40 minutes of simulated ischemia and 18 hours of reperfusion, the cells in 2-chamber slides were fixed by 4% formaldehyde/phosphate-buffered saline at 4°C for 25 minutes and subjected to TUNEL assay according to the manufacturer's protocol. The slides were then counterstained with Vectashield mounting medium with 4,6-diamidino-2-phenylindole (a DNA intercalating dye for visualizing nuclei in fixed cells; catalog number H-1200,

Vector Laboratories, Burlingame, Calif). The stained cells were examined under an Olympus IX70 fluorescence microscope (Olympus Inc, Center Valley, Pa).

Anakinra Uptake In Vitro

HL-1 cells, a mouse cardiac muscle cell line that retains phenotypic characteristics of adult cardiomyocytes, were grown to confluence in Claycomb medium (Sigma Chemical Co) supplemented with 10% FCS (JHR Bioscience, Ltd, Andover, Hampshire, UK) and norepinephrine 0.1 mmol/L (Sigma Chemical Co) on gelatin/fibronectin-coated 22-mm glass coverslips placed on the bottom of 35-mm Petri dishes.[17] FITC-conjugated anakinra (10 μg/mL) was then added to the cells, and the cells were incubated at 37°C in either normoxic or hypoxic (2% oxygen) conditions in a Micro galaxy, RS Biotech incubator for 5 hours. Cells were then washed twice with Hanks' salt solution containing 10% FCS. Nuclei were counterstained with Hoechst, and observations were carried out by a Leica DM 2000 fluorescence microscope (Meyer Instruments Inc, Houston, Tex).

Caspase-1 and -9 Activity Assays

Inhibition by anakinra of caspase-1 and -9 activities was assayed by the commercial assay kits (QuantiZyme assay system, Plymouth, Pa) using recombinant human caspase-1 and YVAD-AMC, a variation on the sequence YVHD found at the pro-Il-1β cleavage site, as fluorogenic substrate for caspase-1 and Ac-LEHD-AMC for caspase-9. The experiment was performed at 22°C with a fluorometric plate reader (SLT, Fluostar, Tecan US, Research Triangle Park, NC) in the kinetic mode with excitation and emission wavelengths of 390 and 460 nm, respectively. A starting concentration of 11.5 μmol/L (equivalent to the Km of caspase-1) was used for the substrate and then increased to 37.5 μmol/L to test for competitive and noncompetitive inhibition.[18] For anakinra, a starting dose of 100 nmol/L in the presence or absence of 10 μg/mL polyclonal anti–IL-1Ra blocking antibody (R&D Systems, Minneapolis, Minn) was chosen and then increased to 300 and 900 nmol/L to evaluate for a dose-response curve. Corrections from blank are performed, and the curves are fitted to a 0,0 intercept because the activity is assumed to be 0 at time 0. Regression slopes ($r>0.95$) are obtained for each experiment at each dose, and coefficients are compared between the 3 groups (anakinra, control, anakinra plus blocking antibody). The experiments were performed in triplicates for each group, and curves were run at least 5 times in each sample. The probability value reflects 1-way ANOVA between groups with 2-sided post-hoc Dunnett's test comparing each group with anakinra.

Physical interaction and binding between anakinra and caspase-1 and -9 were tested by means of coimmunoprecipitation. Anakinra (1 μg) was incubated with commercially available recombinant human caspase-1 and -9 (1 μg, BIOMOL International, Plymouth Meeting, Pa) for 1 hour at 4°C. The mixture was then precipitated by adding it to 2 different sets of Sepharose beads coupled with anti–IL-1Ra (Santa Cruz) or anti–caspase-1 antibody (Santa Cruz). Sepharose-bound fractions were boiled in SDS buffer and separated in SDS-PAGE. The coprecipitated proteins in each setting were then immunoblotted and analyzed by Western blot. Anti–IL-1Ra and anti–caspase-1 or -9 antibodies were used to detect immunoreactivity of anakinra and caspase-1, respectively, in the coprecipitate. If no coprecipitate had occurred, then a single band during Western blot corresponding to the soluble antibody of the same type of the antibody bound to the Sepharose beads (ie, immunostaining for IL-1Ra in the IL-1Ra–bound Sepharose beads precipitation assay) was expected. If coprecipitation had occurred, then we would expect the presence of a dual band for both soluble antibodies in both precipitation assays (4 bands in total). Anakinra and caspase-1 or -9 were run in the Western blot in additional lines and used as positive controls.

Statistical Analysis

Statistical analysis was performed with the SPSS 11.0 package for Windows (SPSS Inc, Chicago, Ill). Continuous variables are expressed as mean and SE. One-way ANOVA was used to compare means between multiple (>2) groups with the post hoc 2-sided Dunnett's test to specifically compare the between-subject effects with controls in each group. The *t* test for unpaired data was used to compare means between 2 groups only. Random-effects ANOVA for repeated measures was used to compare preintervention and postintervention echocardiographic parameters between the 4 different groups with the post hoc 2-sided Dunnett's test to specifically compare the between-subject effects (anakinra and saline AMI groups). Correlations between 2 continuous variables were assessed with Pearson's test. Kaplan-Meier survival curves were constructed, and the log-rank test was used to evaluate for significant differences between groups. Survival rates also were compared by the use of Fisher's exact test. Unadjusted 2-tailed probability values are reported throughout, with statistical significance set at the 0.05 level.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results

The study included the assessment of the effects of anakinra in vivo (in the mouse and rat coronary artery ligation model) and in vitro (in primary rat cardiomyocyte culture, HL-1 cardiomyocyte culture, and isolated caspase-1).

In Vivo Administration of Anakinra

Survival

Twenty-eight mice were euthanized at 24 hours to assess infarct size. Six mice were euthanized at 7 days for MMP-9 expression analysis; these mice were not included in the survival analysis. At 7 days after coronary artery ligation, 15 of the 16 mice (94%) treated with anakinra were alive versus 11 of the 20 mice (55%) treated with saline ($P=0.013$, Kaplan-Meier log-rank test; $P=0.021$, Fisher's exact test). Similarly, 4 of the 4 rats (100%) treated with anakinra were alive, whereas only 5 of the 8 rats (62%) treated with saline were alive. All sham-operated mice (n=8) and rats (n=8) were alive at 7 days.

Infarct Size

No significant differences in risk area and infarct area (expressed as infarct area per risk area) were found between saline-treated mice and mice treated with 1- and 10-mg/kg doses of anakinra; however, anakinra 100 mg/kg was associated with a modest yet significant (13%) reduction in infarct size ($P=0.015$ versus saline; Figure 1).

Apoptosis

Anakinra use was associated with a significant reduction in cardiomyocyte apoptosis in the peri-infarct myocardium in both the immediate and delayed treatment groups (3.1±0.2% versus 0.5±0.3%, $P<0.001$; and 4.2±0.4% versus 1.1±0.2%, $P<0.001$, respectively; Figure 2). The apoptotic rate in the peri-infarct myocardium was directly correlated with signs of adverse remodeling such as LVEDD ($r=0.65$, $P=0.001$), LVESD ($r=0.66$, $P<0.001$), FS ($r=-0.62$, $P=0.001$), anterior wall diastolic thickness ($r=-0.50$, $P=0.012$), and anterior wall systolic thickness ($r=-0.50$, $P=0.012$). The apoptotic rate in the remote myocardium was either undetectable or very low with no significant differences between animals with AMI (0.03±0.03%) and sham-operated animals (0.01±0.01%) and between anakinra-treated (0.02±0.02%) and saline-treated (0.03±0.03%) animals ($P>0.05$ for all analyses).

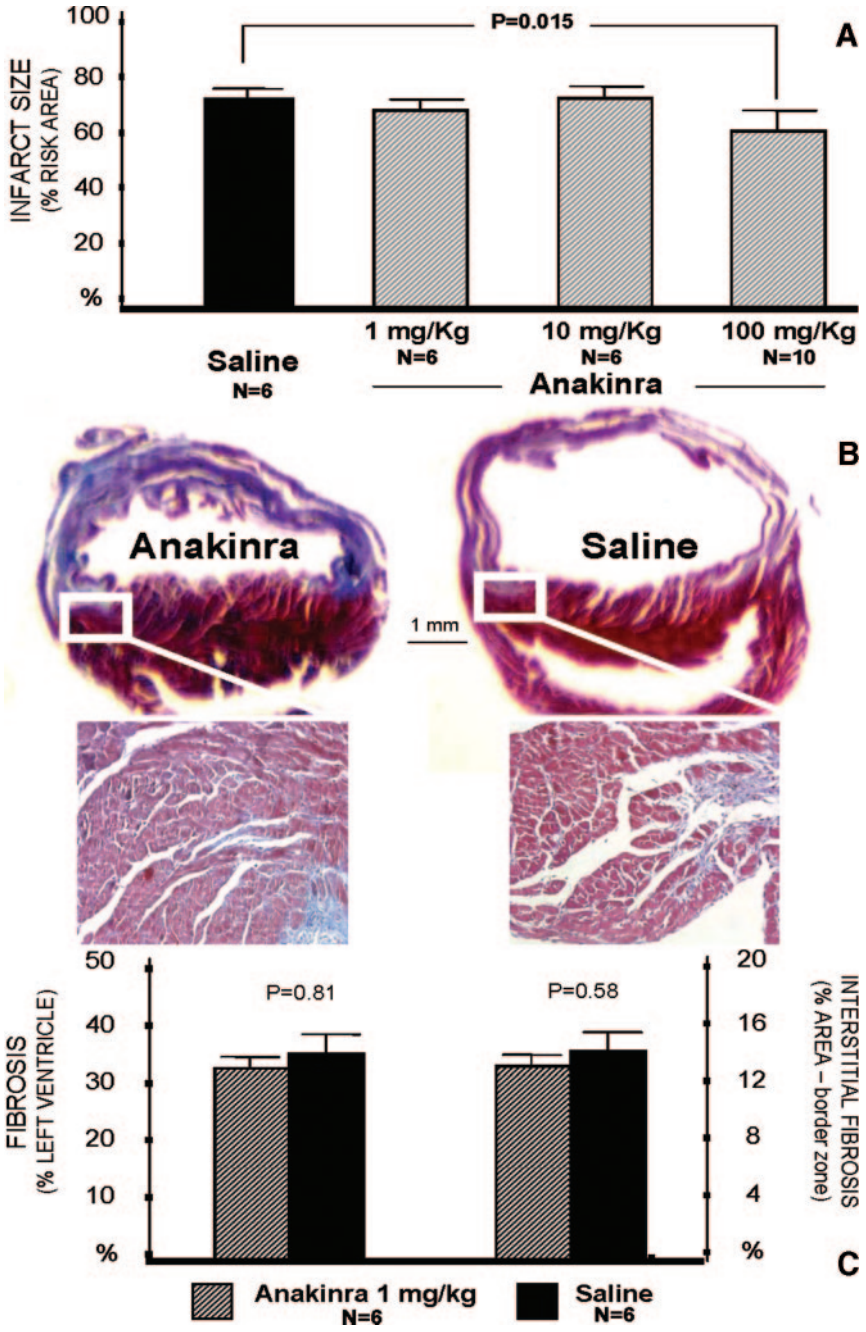

Figure 1. Infarct size. A, Infarct size measurements 24 hours after surgery in mice using triphenyl tetrazolium chloride. No differences in infarct size expressed as percent of risk area at 24 hours were observed with 1- and 10-mg/kg doses, whereas a 13% reduction in infarct size was found with the 100-mg/kg dose ($P=0.015$, 1-way ANOVA between groups with post hoc 2-sided Dunnett's test to specifically compare 100-mg/kg dose and saline). B, Masson's trichrome stains highlight the presence of myocardial fibrosis scar. The enlarged panels show in detail the peri-infarct area in which interstitial fibrosis was measured as percent of surface. C, Quantitative representation of scar and interstitial fibrosis. No differences in myocardial fibrosis (scar formation, expressed as percent of left ventricle) were found between the anakinra- and saline-treated mice. Interstitial fibrosis in the peri-infarct area was minimal in sham-operated mice (<0.1%) and more prevalent in anakinra- and saline-treated mice without any significant differences between groups.

Myocardial Fibrosis and Calcium Deposits

No differences in myocardial fibrosis (scar formation, expressed as percent of the left ventricle) were found between the anakinra- and saline-treated mice (31±2% versus 33±2%; $P=0.81$; Figure 1). Interstitial fibrosis in the peri-infarct area was minimal in sham-operated mice (<0.1%) but more prevalent in anakinra- and saline-treated mice without any significant differences between groups (13±2% versus 15±3%; $P=0.58$; Figure 1). We found minimal amounts of myocardial calcium deposits using von Kossa staining 1 week after AMI (<0.1%) without any differences between anakinra- and saline-treated mice.

LV Remodeling and Function

Preintervention LVEDD and LVESD values were similar in all mouse and rat groups. Significant increases in LVEDD and LVESD and decreases in anterior wall diastolic thickness, anterior wall systolic thickness, and FS in saline-treated mice (versus baseline and sham [saline and anakinra treated]) were observed on day 7 (Figure 3). Compared with saline-treated mice, anakinra-treated AMI mice had significantly smaller LVEDD and LVESD increases and FS decrease on day 7 versus baseline. The decrease in anterior wall systolic thickness also tended to be smaller in anakinra-treated mice (versus saline-treated animals), showing a protective effect in the peri-infarct region, whereas we found no significant differences in posterior wall diastolic thickness (0.96±0.08 versus 0.78±0.11, respectively; $P=0.86$) and posterior wall systolic thickness (1.32±0.08 versus 1.14±0.12, respectively; $P=0.44$). Anakinra-treated mice also had shorter isovolumetric contraction time (6±4 versus 16±5 ms;

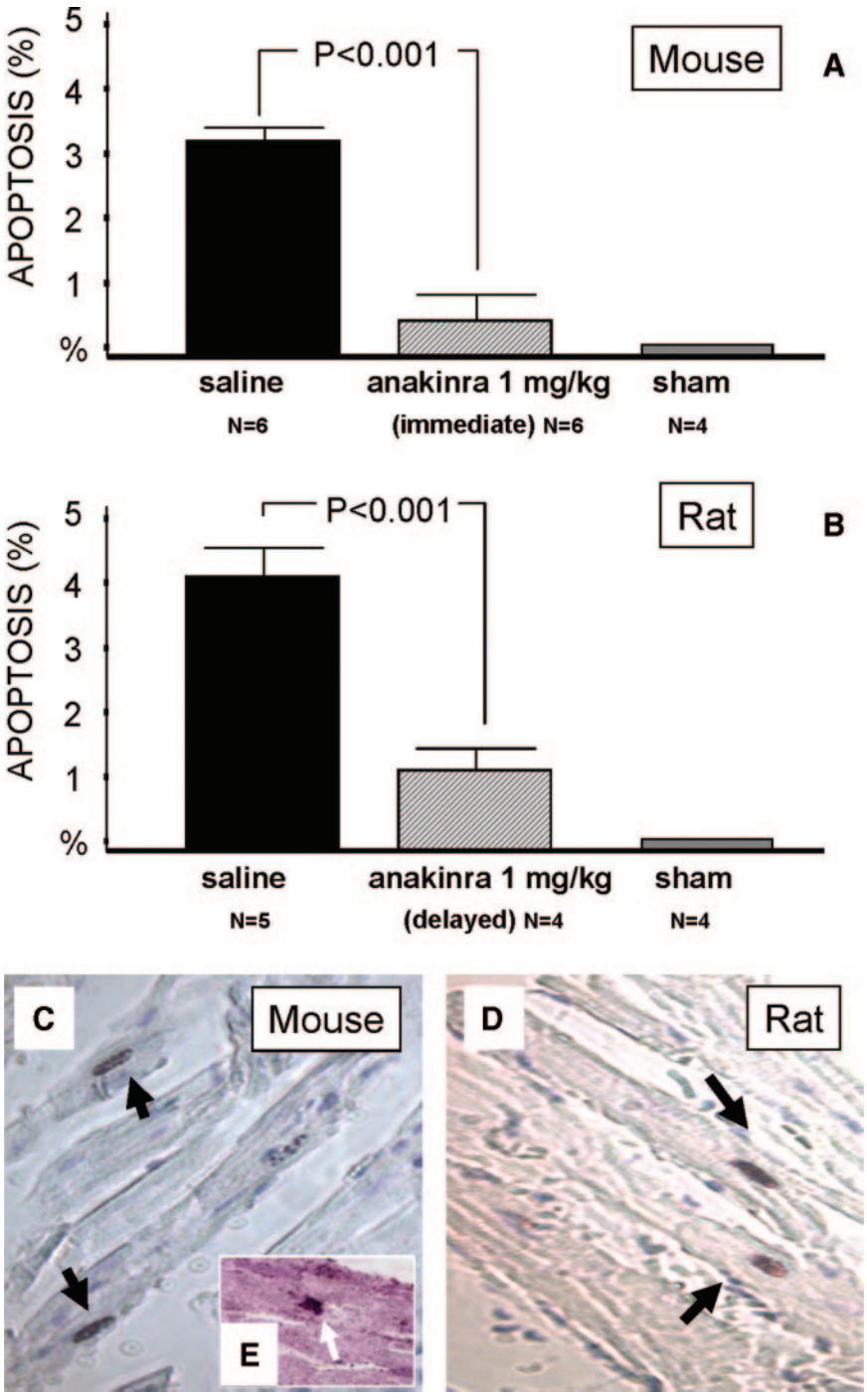

Figure 2. Apoptosis. Apoptotic rates in saline-treated, anakinra-treated, and sham-operated animals are shown. A significantly higher apoptotic rate in saline- vs anakinra-treated AMI animals was found with both immediate anakinra (in the mouse; A) and delayed anakinra group (in the rat; B) ($P<0.001$, 1-way ANOVA between groups with post hoc 2-sided Dunnett's test to specifically compare anakinra and saline in AMI). C, D, Examples of TUNEL-positive cardiomyocytes. E, A double-positive (TUNEL-actin) myocyte.

$P=0.022$) and isovolumetric relaxation time (12±5 versus 29±8 ms; $P=0.042$) values and consequently a lower Tei index (reflecting myocardial performance) (0.28±0.03 versus 0.66±0.07; $P=0.044$; Figure 3). The FS/Tei index, which correlates even more closely to invasive measurements of dP/dt,[19] also was significantly higher in anakinra-treated (0.74±0.06 versus saline-treated 0.17±0.02; $P=0.008$) AMI mice. No differences were found between saline- and anakinra-treated sham-operated mice (Figure 3).

A similar effect on LVEDD and LVESD was seen in the rat model (Figure 4). The mean percent increase in LVEDD and LVESD was significantly greater in mice than rats independently of treatment arm ($P<0.001$). The absolute reduction in LVEDD and LVESD changes with anakinra was significantly greater in mice than in rats ($P<0.001$); however, the percent reductions in LVEDD and LVESD changes were similar in mice (56±6% and 53±5%, respectively) and in rats (68±7% and 47±4%; $P=0.66$ and $P=0.32$, respectively), thus showing similar effects of immediate and delayed anakinra administration.

Leukocyte Infiltration

No differences were found in the number of leukocyte per 1 mm^2 of myocardium in the infarct area in anakinra- and

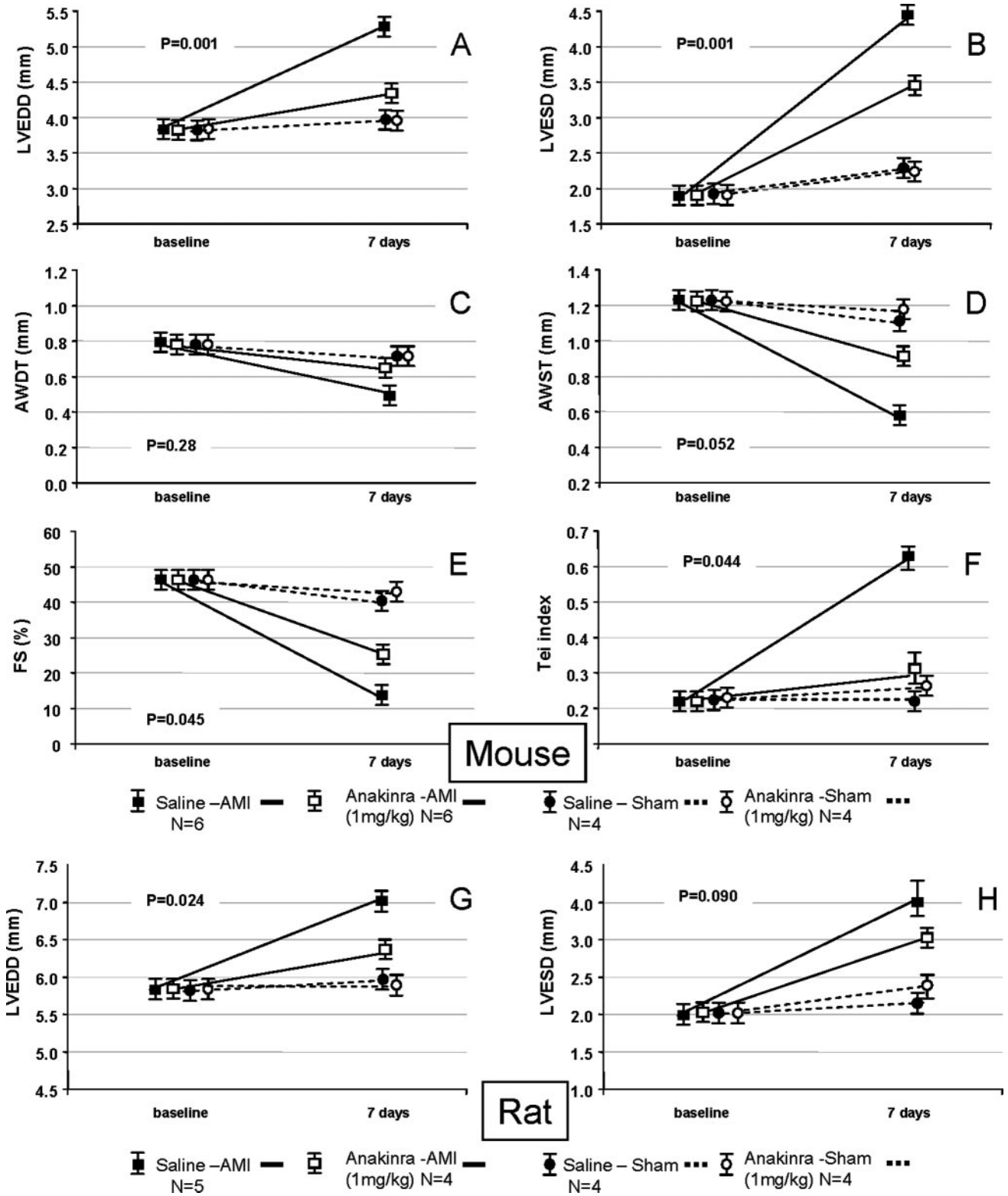

Figure 3. Postinfarction cardiac function and remodeling at echocardiography. Changes in LVEDD, LVESD, anterior wall diastolic thickness (AWDT), anterior wall systolic thickness (AWST), FS, and myocardial performance index (or Tei index) in the mouse model (A through F) and of LVEDD and LVESD in the rat model (G and H) in the saline-treated AMI, anakinra-treated AMI, saline-treated sham-operated, and anakinra-treated sham-operated animals are shown. The probability values shown represent the results of a random-effects ANOVA for repeated measures comparing preintervention and postintervention values between the different groups with the post hoc 2-sided Dunnett's test to specifically compare the between-subject effects (anakinra and saline AMI groups).

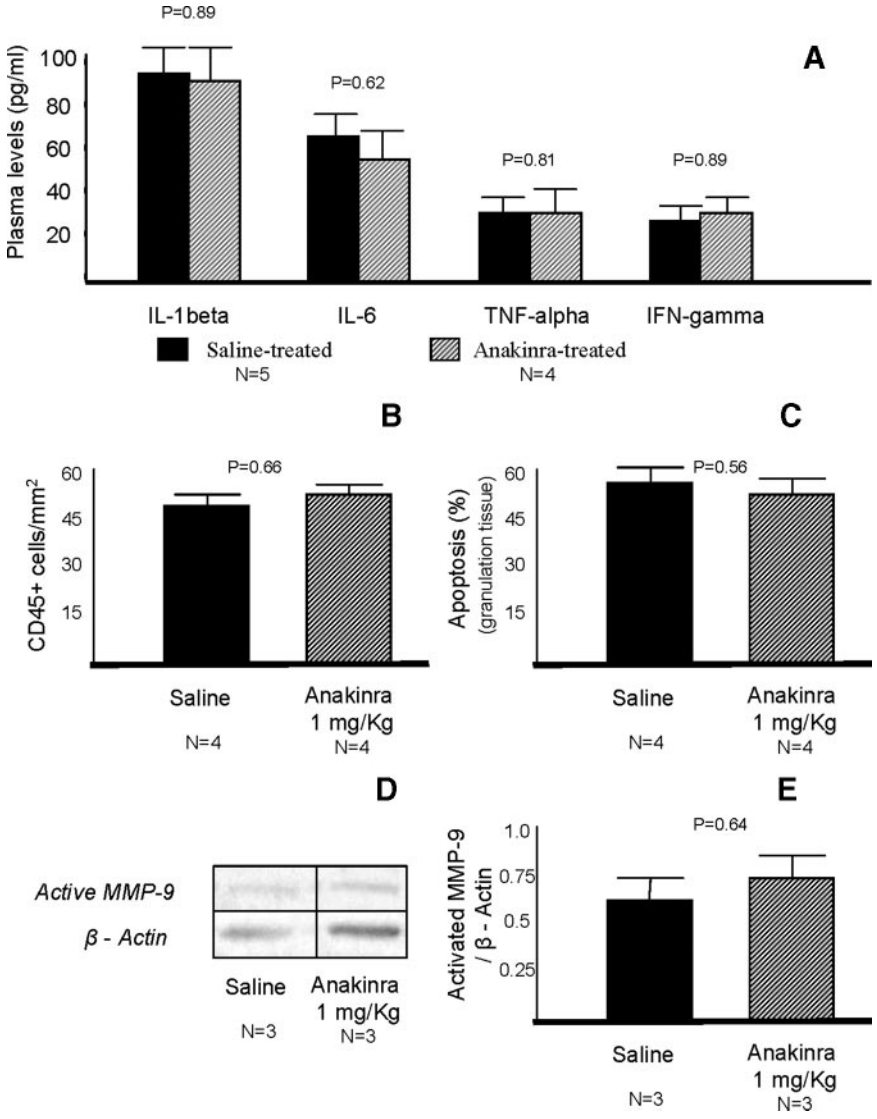

Figure 4. Postinfarction inflammatory response. A, IL-1β, IL-6, tumor necrosis factor (TNF)-α, and interferon (IFN)-γ systemic levels in saline-treated and anakinra-treated AMI rats 7 days after surgery. B, The presence of $CD45^+$ cells in the peri-infarct myocardium in saline-treated and anakinra-treated AMI mice 7 days after surgery. C, The rate of apoptotic (noncardiomyocyte) cells in the granulation tissue in saline- and anakinra-treated AMI mice 7 days after surgery. D and E, Results of Western blot analyses of myocardial proteins from the infarct and peri-infarct regions 7 days after AMI in mice treated with saline or anakinra. No differences in active MMP-9 detection were found between saline- and anakinra-treated AMI mice.

saline-treated AMI animals (Figure 4). The rate of apoptosis in the granulation tissue 1 week after AMI was similar in the anakinra- and saline-treated mice (Figure 4). Leukocytes were virtually absent in remote myocardial regions and in sham-operated animals.

Cytokine Levels

No significant differences were found in IL-1β, IL-6, tumor necrosis factor-α, and interferon-γ plasma levels (Figure 4).

MMP Activity

Active MMP-9 was virtually undetectable in sham-operated animals, whereas it was consistently detected in mice with AMI 7 days after surgery. No difference in active MMP-9 levels was found between saline- and anakinra-treated AMI mice (Figure 4).

In Vitro Administration of Anakinra

Apoptosis

Incubation of cardiomyocytes with anakinra (2.5×10^{-12} g/mL) at the time of "simulated reperfusion" (after 40 minutes of "simulated ischemia") was associated with a significant 36% reduction in apoptosis (11.2±0.5% versus 17.5±0.1% in control). Increasing (×100) concentrations of anakinra up to 25×10^{-6} g/mL showed no additional reduction in apoptosis, whereas concentrations below 25×10^{-12} g/mL had no effect on apoptosis (Figure 5).

Increased Cellular Uptake of Anakinra During Hypoxia

Compared with normoxia, uptake of anakinra during hypoxia was significantly increased, being evident in ≈95% of cells during hypoxia (versus 35% during normoxia; $P=0.048$; Figure 6).

Inhibition of Caspase-1 and -9 Activities

In vitro, anakinra (100 to 900 nmol/L) significantly inhibited caspase-1 and -9 activities by ≈50% ($P<0.001$ for all concentration values versus control), without any differences between different concentrations. Anakinra behaved like a mixed competitive and noncompetitive enzyme inhibitor for caspase-1 (K_i, 0.201 μmol/L; K_{ic}, 0.239 μmol/L; K_{uc}, 0.231 μmol/L) and for caspase-9 (K_i, 0.31 μmol/L; K_{ic}, 0.34 μmol/L; and K_{uc}, 0.28 μmol/L). Addition of IL-1Ra–block-

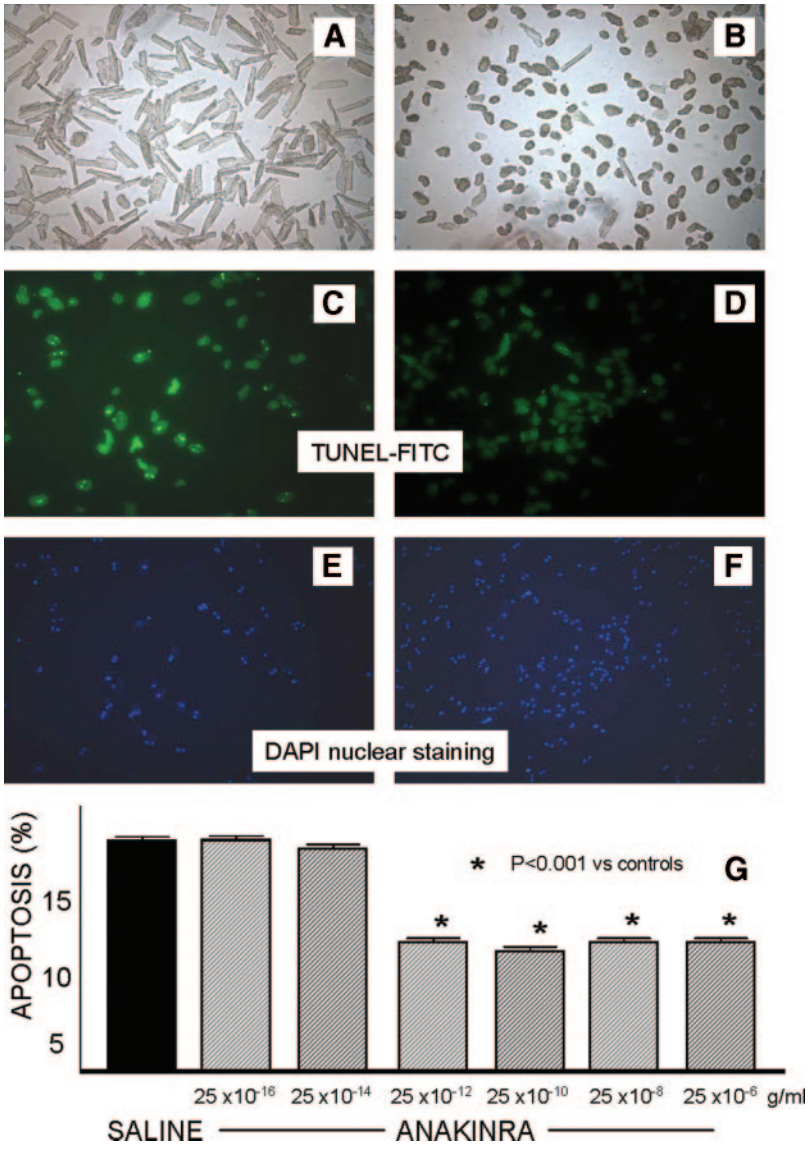

Figure 5. Apoptosis (in vitro study). A and B, Isolated rat cardiomyocytes in culture in normoxic and hypoxic conditions, respectively. C through F, TUNEL-FITC and DAPI in saline—treated and anakinra treated cells, respectively. Incubation of cardiomyocytes with anakinra (2.5×10^{-12} g/mL) at the time of "simulated reperfusion" (after 40 minutes of "simulated ischemia") was associated with a significant 36% reduction in apoptosis (11.2 ± 0.5 versus 17.5 ± 0.1 without anakinra). Increasing ($\times100$) concentrations of anakinra up to 25×10^{-6} g/mL showed no additional reduction in apoptosis, whereas concentrations $<25\times10^{-12}$ g/mL had no effects on apoptosis (G). Probability values reflect results of a 1-way ANOVA test between groups with post hoc 2-sided Dunnett's test to specifically compare different doses of anakinra and saline).

ing antibodies reversed the caspase-1 and -9 inhibition by anakinra (Figure 7). The coprecipitation assay confirmed a physical interaction and binding between anakinra and caspase-1 and -9 (Figure 7).

Discussion

This study shows for the first time that the exogenous recombinant human IL-1Ra anakinra given within the first 24 hours of AMI significantly ameliorates cardiac remodeling by reducing cardiomyocyte apoptosis in 2 different animal models of permanent infarct-related artery occlusion and that anakinra has a direct antiapoptotic effect on cardiomyocytes in vitro.

Biology of the IL-1Ra

IL-1Ra is considered an acute-phase reactant.[3,4] Currently, the role of endogenous IL-1Ra in inflammation is unclear. IL-1Ra binds to the IL-1 receptor and therefore is a competitive inhibitor of IL-1 activity, potentially behaving as an antiinflammatory agent.[3] However, because IL-1 binds its receptor with higher affinity and there is an excess of receptors (spare receptor effect), the role of the endogenous agonist appears to be limited.[3] The IL-1Ra gene is well preserved in biology, and interspecies differences have not been reported. In this study, we tested the 2 most common rodent species and reported similar effects.

IL-1Ra in AMI

IL-1Ra levels increase significantly after myocardial ischemia-reperfusion.[7] Its levels are elevated early in patients presenting with ST-segment-elevation AMI,[5] and the greater the area of myocardium at risk is, the greater the increase in IL-1Ra levels is.[6] Moreover, in observational studies, higher IL-1Ra levels in patients with acute coronary syndromes were associated with unfavorable outcome.[20,21] Whether IL-1Ra represented a marker of damage, an attempt at cardioprotection by means of antiinflammatory activity, or a mediator of damage remained unclear. Overexpression of IL-1Ra in a rat model of global ischemia-reperfusion showed for the first time a cardioprotective effect of IL-1Ra, resulting in an approximate 50% reduction in cardiomyocyte apoptosis.[8]

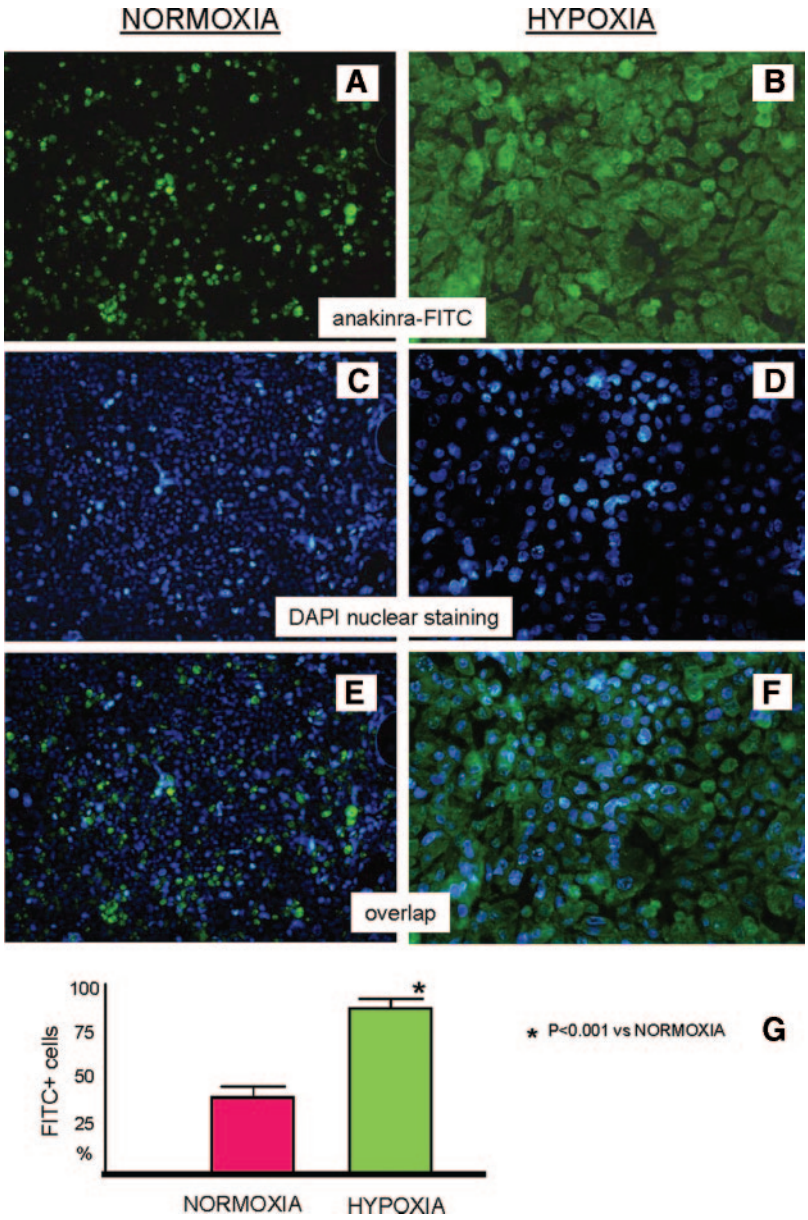

Figure 6. Uptake of anakinra in normoxic and hypoxic conditions. A through F, Anakinra-FITC, DAPI, and overlap in normoxic and hypoxic conditions. Compared with normoxia, uptake of anakinra during hypoxia was significantly increased during hypoxia (G).

Exogenous IL-1Ra

A recombinant human IL-1Ra (anakinra) is commercially available (produced by Amgen), approved by the Food and Drug Administration, and used in a large number of patients for the treatment of rheumatoid arthritis.[22–24] The role of IL-1 in the pathogenesis of rheumatoid arthritis is central. Systemic administration of anakinra has been shown to be safe and effective in patients with rheumatoid arthritis, leading to disease activity modification. A clinical trial of anakinra in patients with acute coronary syndromes is ongoing, but results are not yet available.[25] In a recently published phase II study, 17 patients with ischemic stroke were treated with anakinra given as a 100-mg bolus followed by a 72-hour infusion; the other 17 patients received matching placebo.[26] Anakinra was found to have no drug-related adverse effects and, in a secondary analysis, was found to be associated with a greater number of patients with minimal or no stroke-related disability. In experimental animal stroke models, anakinra was associated with reduced apoptosis, reduced inflammation, and improved behavioral outcome.[27,28]

Mechanism of Action

The exact mechanism by which anakinra exerts its beneficial effects is not completely clear. The accepted view is that anakinra administered in high doses competes with IL-1 and reduces IL-1 activity. Anakinra binds the type I IL-1 receptor but prevents transduction of the intracellular signal by preventing the interaction between the IL-1 receptor and the IL-1 accessory protein. IL-1Ra has been shown to reduce IL-1–dependent prostaglandin-E2 secretion, which may be directly responsible for cell toxicity and apoptosis.[3,29] Whether anakinra interferes with IL-1 derived mainly from inflammatory cells or with IL-1 released locally in a paracrine or autocrine fashion and whether it has a direct effect on the cell independently of its interaction on the IL-1 receptor remain unclear. The intracellular action of IL-1Ra is independent of

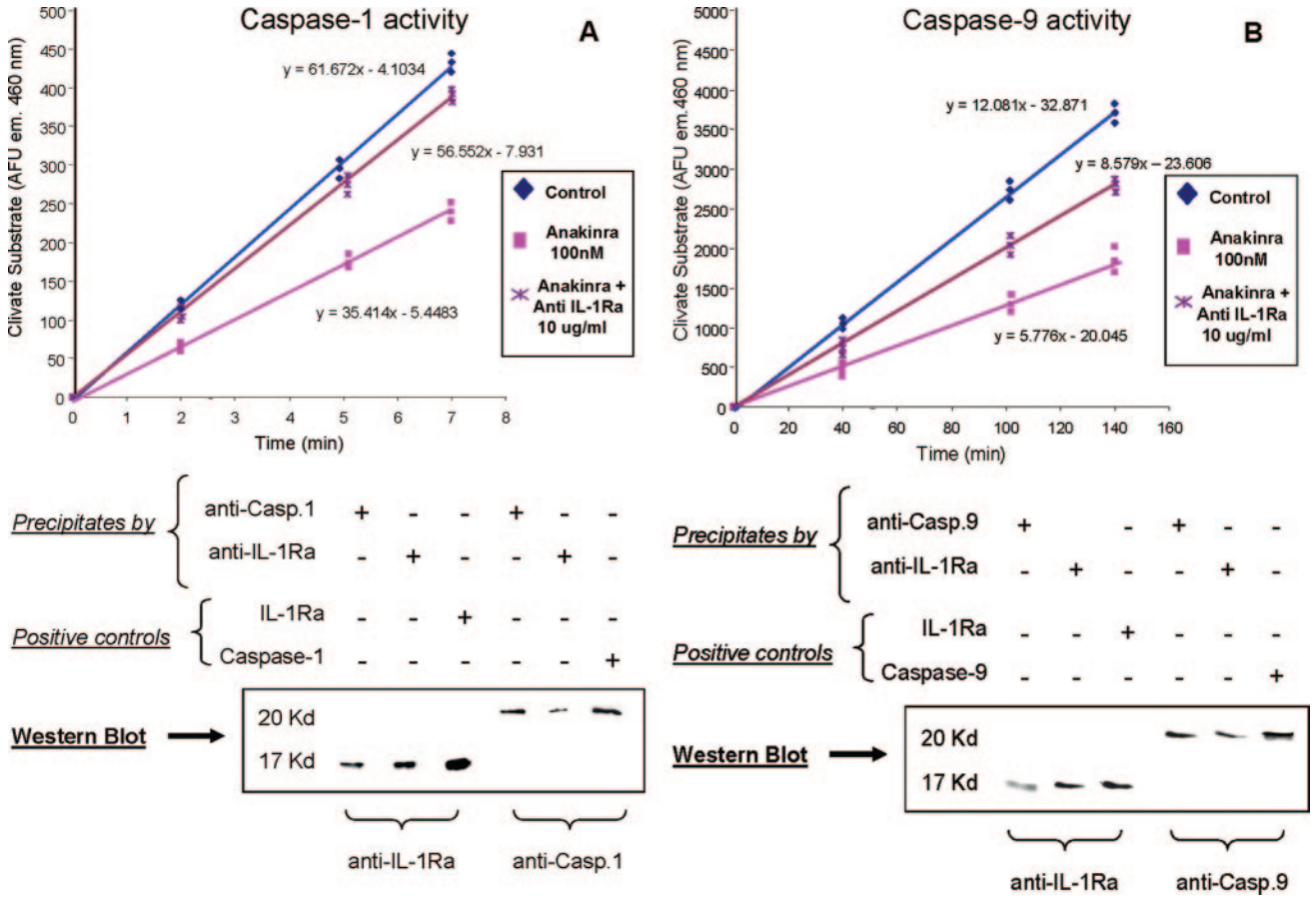

Figure 7. Caspase-1 and -9 inhibition (in vitro study). A and B, In vitro anakinra 100 nmol/L significantly inhibits caspase-1 and -9 activities, respectively, by ≈50% (data shown represent mean values in triplicate samples; $P<0.001$ for anakinra 100 nmol/L vs control for both experiments). Addition of IL-1Ra–blocking antibodies partially reverses the caspase-1 and -9 inhibition by anakinra ($P=0.80$ for anakinra plus antibody vs anakinra for caspase-1; and $P=0.12$ for caspase-9). Physical interaction (A) and binding (B) between anakinra and caspase-1 and -9 are shown by means of coimmunoprecipitation. Anakinra was incubated with commercially available recombinant human caspase-1 or -9. The mixture was then precipitated by adding it to 3 different sets of Sepharose beads coupled with anti–IL-1Ra, anti–caspase-1, or anti–caspase-9 antibody. The coprecipitated proteins in each setting were then analyzed by Western blot. Anti–IL-1Ra and anti–caspase-1 or -9 antibodies were used to detect immunoreactivity of anakinra and caspase-1 or -9, respectively, in the coprecipitate. Coprecipitation is documented by the presence of a dual band for both soluble antibodies in both precipitation assay (lines 1 to 2 and 4 to 5, respectively, for caspase-1; lines 7 to 8 and 10 to 11 for caspase-9). Anakinra and caspase-1 or -9 were run in the Western blot in additional lines (lines 3 and 9 and lines 6 and 12, respectively) and used as positive controls.

the IL-1 intracellular signaling pathway,[30] and transmembrane active transport of IL-1Ra through the $P2X_7$ purinergic channel receptor has been described.[31] The effects observed in vitro where only cardiomyocytes are cultured suggest that the effects of anakinra on myocardium are, at least in part, independent of the presence of an inflammatory infiltrate. Accordingly, we found no effects of anakinra on circulating cytokine levels, myocardial leukocyte infiltrate, or MMP-9 activity in this experimental model. The antiapoptotic effects of the IL-1Ra have already been reported in neurons and epithelial cells.[32,33] These effects are evident in vivo and in vitro, suggesting a paracrine or autocrine effect of IL-1Ra.[32,33] Here, we report cellular uptake by anakinra during ischemia, binding between anakinra and caspase-1 and -9, and significant inhibition of caspase-1 and -9 activities by anakinra. The data obtained in vitro, although only hypothesis generating regarding the events in vivo because of the inherent limitations of the models, suggest an increasing threshold to hypoxia-induced apoptosis with anakinra.

We show that early (immediate) or delayed (24 hours later) administration of anakinra reduces apoptosis and prevents cardiac dilatation after AMI. At the dose shown to inhibit apoptosis and prevent dilatation (1 mg/kg), anakinra had no effect on infarct size when given early, and similar benefits in remodeling are seen with anakinra administered 24 hours after AMI, in keeping with an effect on cardiac remodeling that is independent of infarct sparing. Notably, the beneficial effects of IL-1Ra in animal stroke models are also, at least in part, time independent.[34] However, as already noted in the stroke literature, an infarct-sparing effect was observed with a 100-fold-higher dose of anakinra. Whether the small yet significant reduction in infarct size with higher doses would translate to a clinically relevant benefit in terms of heart failure or survival is unknown and requires further testing.

The optimal duration of IL-1Ra treatment after AMI is unknown. In a model of vessel wall response to injury, IL-1Ra given for 28 days was associated with a greater effect compared with IL-1Ra given for 14 days.[35] Of note, no

rebound effects were found after discontinuation of IL-1Ra treatment.[35]

Role of Caspases in Ischemia and Heart Failure

Caspases are proteases involved in the apoptotic and inflammatory cascade.[36–40] Caspase-3 is a central mediator of the apoptotic cascade, leading to downstream activation of secondary effectors responsible for DNA fragmentation and cleavage of cytoskeleton structural proteins. Caspase-1, also known as the interleukin-1–converting enzyme, is considered a proinflammatory caspase because, among its actions, it converts pro–IL-1β into IL-1β. Caspase-1, however, also is one of the enzymes that cleave pro–caspase-3, leading to its activation.[36–40] Indeed, caspase-3 is generally activated from cleavage by other caspases such as caspase-9, which plays a central role in the mitochondrial pathway; caspase-8, which is involved mostly in receptor-mediated apoptosis; and caspase-1. Experimental studies have shown that inhibition of caspase-1 activity is associated with reduced apoptosis and more favorable remodeling after AMI independently of IL-1 levels.[38,39] Considering that IL-1 is the substrate for caspase-1, we tested whether IL-1Ra could exert its beneficial effects by direct intracellular inhibition of caspase-1.[40] Accordingly, we describe, for the first time, that anakinra binds to caspase-1 in vitro and significantly inhibits its activity. Direct inhibition of caspase-1 by anakinra may be responsible, at least in part, for its antiinflammatory and antiapoptotic effects. Anakinra, however, may have antiapoptotic effects resulting from an indirect effect on caspase-1 activity by preventing translocation of caspase-1 to the nucleus and therefore inhibiting apoptosis[41] or through inhibition of caspase-9. Caspase-9 is the key mediator of the mitochondrial pathway, leading to activation of apoptosis during hypoxia/ischemia.[37] Whether inhibition of caspase-1 or -9 plays a greater role in the benefit observed with anakinra remains to be elucidated. At the dose tested in this study, anakinra likely had similar inhibitory effects on both caspases, and crosstalk between different caspases in the apoptotic cascade has been reported.[36–41]

Pharmacokinetics of Anakinra and Dose-Effect Response

In a phase I study in 25 healthy volunteers, a single dose of anakinra at a dose similar to that used in this study (1 mg/kg) administered intravenously was associated with a plasma level of 3.1 μg/mL and a half-life of 2.64 hours.[42] In vivo, we found no significant effects on infarct size using anakinra at a dose up to 10 times higher than the recommended dose, but we found a small yet significant reduction in infarct size using a dose 100 times higher. Whether this infarct-sparing effect associated with such a high dose would translate to a favorable long-term outcome is uncertain and requires further studies. In vivo, a concentration $>10^6$ lower than the peak plasma level observed in humans was associated with a significant reduction in apoptosis. Lower doses were not effective, whereas higher doses showed no additive effects. These data are in keeping with the clinical profile of anakinra in which a standard dose of 1 mg/kg is generally used and larger doses are associated with more local adverse reactions without significant additional clinical benefit.[22–24] In a recent study,[43] anakinra given at a dose of 100 mg (grossly equivalent to 1 mg/kg) was used to inhibit pancreatic β-cell apoptosis and was shown to be well tolerated and to be associated with indexes of better β-cell function compared with placebo without affecting insulin sensitivity.

Ischemia, Apoptosis, and Heart Failure After AMI

The finding of an association between apoptosis and remodeling supports the concept of apoptosis as a central mediator of cardiac remodeling regardless of infarct size.[1,37,44] The lack of differences in its effects on apoptosis or remodeling between the immediate and delayed treatment strategy and the lack of effects on infarct size with the 1-mg/kg dose suggest that anakinra specifically affects subacute postinfarction remodeling, in which apoptosis is known to play a major role,[1,36,43] without affecting infarct healing, matrix degradation, and fibrosis or promoting wall rupture.

Conclusions

Administration of anakinra within 24 hours of AMI ameliorates postinfarction remodeling while inhibiting apoptosis. Despite the limitations of the present study (such as a relative small sample size limited to male gender, a single time determination of outcomes of interest, and the use of echocardiography, which remains a suboptimal and operator-dependent method for assessing LV remodeling and function), the findings of a beneficial effect of exogenous IL-1Ra (anakinra) given within 24 hours of AMI may open a new therapeutic window for the treatment of ischemic injury and remodeling for the prevention and treatment of ischemic heart failure.

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Disclosures

None.

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CLINICAL PERSPECTIVE

Acute myocardial infarction remains a major cause of morbidity and mortality despite current strategies for early reperfusion. Many patients die early during the course, and those who survive are at risk of dying late as a result of adverse cardiac remodeling and heart failure. The initial ischemic damage to the myocardium activates reparative events that are initially beneficial but entrain late events that eventually lead to adverse cardiac remodeling and heart failure. Ventricular remodeling is a complex process mediated by alterations in stereotypic pathways that can trigger hypertrophy and apoptosis (programmed cell death), resulting in a delicate balance between cardiomyocyte death and survival. Interventions that limit the extent of infarction and/or inhibit apoptosis have the potential to prevent adverse cardiac remodeling and heart failure–related deaths. Interleukin-1 (IL-1) receptor antagonist (IL-1Ra) is part of the IL-1 family and competes with IL-1β for its receptor acting as an antiinflammatory protein. IL-1Ra levels are consistently elevated in patients with acute myocardial infarction and behave as a marker of disease. Using an established model of acute myocardial infarction with surgical ligation of the left coronary artery, we have found that IL-1Ra promotes cell survival through an intrinsic antiapoptotic activity. Administration of anakinra, an exogenous recombinant human IL-1Ra, within 24 hours of acute myocardial infarction in this model resulted in amelioration of postinfarction cardiac remodeling and heart failure. These findings may open a new therapeutic window for the treatment of ischemic injury and remodeling for the prevention and treatment of ischemic heart failure, which may ultimately benefit several thousands of patients.

Anakinra, a Recombinant Human Interleukin-1 Receptor Antagonist, Inhibits Apoptosis in Experimental Acute Myocardial Infarction

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