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Research Article

The human heart is a target organ for the octapeptide hormone, angiotensin II (Ang II). Recent studies suggest that the human heart contains a dual pathway of Ang II formation in which the major Ang II-forming enzymes are angiotensin I-converting enzyme (ACE) and chymase. Human heart chymase has recently been purified and its cDNA and gene cloned. This cardiac serine proteinase is the most efficient and specific Ang II-forming enzyme described. To obtain insights into the cardiac sites of chymase-dependent Ang II formation, we examined the cellular localization and regional distribution of chymase in the human heart. Electron microscope immunocytochemistry using an anti-human chymase antibody showed the presence of chymase-like immunoreactivity in the cardiac interstitium and in cytosolic granules of mast cells, endothelial cells, and some mesenchymal interstitial cells. In the cardiac interstitium, chymase-like immunoreactivity is associated with the extracellular matrix. In situ hybridization studies further indicated that chymase mRNA is expressed in endothelial cells and in interstitial cells, including mast cells. Tissue chymase levels were determined by activity assays and by Western blot analyses. Chymase levels were approximately twofold higher in ventricles than in atria. There were no significant differences in chymase levels in ventricular tissues obtained from non-failing donor hearts, failing ischemic hearts, or hearts from patients with ischemic cardiomyopathy. These findings suggest that a major site of [...]

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Cellular Localization and Regional Distribution of an Angiotensin II-forming Chymase in the Heart

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Abstract

The human heart is a target organ for the octapeptide hormone, angiotensin II (Ang II). Recent studies suggest that the human heart contains a dual pathway of Ang II formation in which the major Ang II-forming enzymes are angiotensin I-converting enzyme (ACE) and chymase. Human heart chymase has recently been purified and its cDNA and gene cloned. This cardiac serine proteinase is the most efficient and specific Ang II-forming enzyme described. To obtain insights into the cardiac sites of chymase-dependent Ang II formation, we examined the cellular localization and regional distribution of chymase in the human heart. Electron microscope immunocytochemistry using an anti-human chymase antibody showed the presence of chymase-like immunoreactivity in the cardiac interstitium and in cytosolic granules of mast cells, endothelial cells, and some mesenchymal interstitial cells. In the cardiac interstitium, chymase-like immunoreactivity is associated with the extracellular matrix. In situ hybridization studies further indicated that chymase mRNA is expressed in endothelial cells and in interstitial cells, including mast cells. Tissue chymase levels were determined by activity assays and by Western blot analyses. Chymase levels were approximately twofold higher in ventricles than in atria. There were no significant differences in chymase levels in ventricular tissues obtained from nonfailing donor hearts, failing ischemic hearts, or hearts from patients with ischemic cardiomyopathy. These findings suggest that a major site of chymase-dependent Ang II formation in the heart is the interstitium and that cardiac mast cells, mesenchymal interstitial cells, and endothelial cells are the cellular sites of synthesis and storage of chymase. In the human heart, because ACE levels are highest in the atria and chymase levels are highest in ventricles, it is likely that the relative contribution of ACE and chymase to cardiac Ang II formation varies with the cardiac chamber. Such differences may lead to differential suppression of cardiac Ang II levels during chronic ACE inhibitor therapy in patients with congestive heart failure. (*J. Clin. Invest.* 1993. 91:1269–1281.) Key words: angiotensin I-converting

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Introduction

In blood, angiotensin II (Ang II)¹ is produced by the sequential processing of angiotensinogen by renin and by angiotensin I-converting enzyme (ACE) (1). Ang II is a vasoconstrictor hormone (1), and drugs that inhibit ACE are potent antihypertensive agents (2). ACE inhibitors are also very effective in the treatment of congestive heart failure (3, 4). It is generally believed that the vasodilator effect of ACE inhibitors that produces a marked unloading of the failing heart is paramount to its beneficial effect (5). Recently, some investigators have proposed that direct cardiac effects of ACE inhibitors may also be therapeutically important (6).

Numerous studies have indicated that Ang II has multiple actions on the heart (7). It produces a positive inotropic and chronotropic effect by a direct action on cardiac myocytes and by stimulating the release of norepinephrine from cardiac sympathetic nerves. Consistent with these effects of Ang II, we have shown that Ang II receptors are present on atrial and ventricular myocytes and on cardiac sympathetic nerves in human heart tissue (8). Others have shown that nanomolar concentrations of Ang II produce a positive inotropic effect on isolated human cardiac trabeculae (9, 10). Studies on cells in culture show that Ang II is a cardiac growth factor (11). These studies clearly suggest that the human heart is a target organ for Ang II.

Paracrine regulation of human heart function by Ang II has been proposed, but the enzymatic pathways of Ang II formation in the human heart are only now being addressed (12, 13). Such studies are important in understanding the mechanism of action of ACE inhibitors in patients with congestive heart failure. Our recent studies indicate that the human cardiac ventricles contain a dual enzymatic pathway for Ang II formation in which ACE-dependent Ang II formation is minor (~10%) compared to a major serine proteinase-dependent Ang II formation (~80%) (12). We have isolated and sequenced this serine proteinase, a novel enzyme that is not inhibited by ACE inhibitors, and we have cloned its cDNA and gene (13, 14). This novel proteinase is the most efficient and specific Ang II-forming enzyme described (13, 15) and is the first structurally characterized human member of the chymase group of enzymes. To gain insights into factors that may regulate chymase expression and to delineate potential sites of chymase-dependent Ang II formation in the heart, we examined the regional distribution and the subcellular localization of human

1. Abbreviations used in this paper: Ang, angiotensin; ACE, angiotensin I-converting enzyme; EM, electron microscopy.

heart chymase. Our results indicate that the cardiac interstitium may be an important site of Ang II formation in the human heart, and that several cell types, including the cardiac mast cell and endothelial cell, are the sites of biosynthesis and storage of chymase. Because Ang II has also been implicated in the pathogenesis of congestive heart failure, we have additionally examined levels of chymase-like activity and chymase mRNA in normal and failing human hearts.

Methods

Human tissue. Nonfailing donor hearts ($n = 13$) were obtained from LifeBanc (Cleveland, OH). These hearts, from 10 men and 3 women ranging in age from 16 to 48 yr, were unsuitable for donation. Diseased human hearts ($n = 15$), excised from patients undergoing cardiac transplantation at the Cleveland Clinic Foundation, were the source of failing heart tissue. Each of these diseased hearts was from a patient with end-stage congestive heart failure accompanied by underlying coronary artery disease; the patients were characterized as either ischemic ($n = 5$; four male and one female, 39 to 57 yr), or dilated idiopathic cardiomyopathy without active myocarditis ($n = 10$, seven males and three females). All heart tissue was kept in cold cardioplegia solution from the time of removal and frozen within 2 h. Healthy human tissue including lung, liver, skin, kidney, and spleen were obtained from various patients undergoing surgical procedures at the Cleveland Clinic Foundation. The use of these tissues was approved by The Cleveland Clinic Foundation's Internal Review Committee, and consent was obtained from the donor or from his/her relatives or legal next of kin.

Electron microscopy (EM) immunocytochemistry. EM immunocytochemistry was carried out according to the method of Wolosewick et al. (16) with slight modification. Small pieces (0.5 cm cubic) of tissue obtained from one nonfailing donor and one ischemic failing heart were fixed within 2 h from the excision in 0.1 M cacodylate buffer, pH 7.3, containing 4% paraformaldehyde at 4°C overnight. Tissues were further fixed in the same buffer containing 4% paraformaldehyde and 3% acrolein for 10 min. Then the samples were postfixured in the same buffer containing 4% paraformaldehyde and 0.25% glutaraldehyde for 1 h. The samples were washed and dehydrated by a graded series $\leq 80\%$ ethanol. The samples were infiltrated, embedded in acrylic resin (LR White; Electron Microscopy Sciences, Fort Washington, PA), and blocked out in gelatin capsules. Blocks were polymerized for 48 h at 50°C. A tissue block was sectioned on an RMC 6000 ultramicrotome, and sections were mounted on 200 mesh formvar supported nickel grid. Immunolabeling experiments and controls were repeated four times with ventricular tissue and two times with atrial tissue. Over 20 grids were examined. The surface of the sections was first etched in 1 ml freshly prepared saturated sodium metaperiodate at 22°C for 15 min. This was followed by a rinse in distilled water, an exposure for 10 min to 1 ml of 0.1N HCl, and another rinse in distilled water. Sections on the grids were then exposed to 0.5 M ammonium chloride in 1 ml of PBS, pH 7.4, for 30 min. These grids were preincubated in 600 μ l of 20 mM Tris-HCl, pH 8.2, containing 0.1% BSA and 5% normal goat serum. They were incubated with the diluted (100 \times) antiserum against human heart chymase (13) or with the diluted preimmune serum (100 \times) in the same buffer containing 0.1% BSA and 1% normal goat serum at 22°C overnight. After several washes in the same buffer, these grids were incubated with 10 nm gold conjugated goat anti-rabbit antibody (GAR IgG Au10; Amersham Corp., Arlington Heights, IL) diluted (30 \times) in the same buffer at 22°C for 3 h. At the end of incubation these grids were washed several times by the same buffer and extensively washed by distilled water. Samples on the grids were contrasted with 1% uranyl acetate (Electron Microscopy Sciences) and were examined and photographed in an electron microscope (model EM10; Carl Zeiss, Inc., Thornwood, NY) at 60 KV with an objective aperture of 30 μ m for photographs. Astigmatism caused by the magnetism of the nickel grids was checked and adjusted for each grid.

In situ hybridization. Human heart tissues were fixed in 2% paraformaldehyde in $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free PBS, pH 7.4, and 5- μ m paraffin sections were prepared on poly-L-lysine-pretreated microslides. 10 sections from each chamber of one donor heart from a 26-yr-old male and one recipient heart from a 34-yr-old male with idiopathic cardiomyopathy were examined. An antisense oligonucleotide DNA probe corresponding to a unique 13-residue sequence of human chymase (+17–+29; see reference 14) and its corresponding sense probe were synthesized: CTACCTGGAAATTGTAACCTCCAACGGTCCCTCAAATTT, 126–165 bp, sense-strand; AAATTTTGGAGGGACCGTTGGAAGTTACAATTTCCAGGTAG, 165–126 bp, antisense strand in the human heart chymase cDNA (14). After purification, probes were 5' end labeled with γ [^{32}P] ATP (specific activity $\approx 1 \times 10^9$ dpm/ μ g DNA). Hybridization was performed as previously described (17). Sections were incubated in 20 mM Tris-HCl, pH 7.4, containing 1 μ g/ml of proteinase K (type 28; Sigma Chemical Co., St. Louis, MO) and 2 mM CaCl_2 for 30 min at 37°C after removal of paraffin with xylene. The tissue section was treated with 0.1 M triethanolamine, pH 8.0, for 2 min at 22°C and with 0.25% (vol/vol) acetic anhydride in 0.1 M triethanolamine, pH 8.0, for 10 min at 22°C. The radiolabeled probes were incubated with the tissue sections (5 ng/section) in 10 mM Tris-HCl buffer, pH 7.5, containing 300 mM NaCl, 1 mM EDTA, 70% formamide (vol/vol), 2% dextran sulfate, 250 μ g/ml salmon testes DNA, and 250 μ g/ml yeast transfer RNA for 20 hr at 42°C. The sections were then washed twice in 0.3 M NaCl/30 mM sodium citrate for 30 min at 45°C and once for 60 min at 50°C. Slides were then coated with Ilford K.2 nuclear track emulsion and left at -20°C for 3 wk. Autoradiographic grains were developed using Kodak D-19, and the tissue was counterstained with hematoxylin and eosin Y. Adjacent sections were also stained by Malloney's alcian blue method (18). Radiolabeled oligonucleotide sense probe was also used as an indicator of background and nonspecific binding.

Southern blot analyses of human genomic DNA. Southern blot analyses of human genomic DNA were carried out by a method previously reported (14). 7 μ g of each leukocyte DNA prepared from three healthy volunteers was digested by the restriction enzymes EcoRI, EcoRI + XbaI, RsaI, PstI, PvuII, HindIII, or SacI, and the resulting fragments were resolved by 0.7% agarose gel electrophoresis. Fragments that hybridized the full-length human heart chymase cDNA probe that was ^{32}P -labeled by random priming were then identified by Southern blot analyses. To identify the additional fragment, if any, which is not expected based on the restriction map of the human chymase gene, the analyses were performed under low stringent wash conditions (2 \times SSC, 0.1% SDS, 45°C). The patterns of restriction fragments of each blot from three different normal healthy volunteers were compared.

Northern blot analyses. Poly (A) $^+$ RNA was isolated from 1 g of human heart tissue using a poly (A) $^+$ RNA extraction kit (Fasttrack; Invitrogen, San Diego, CA). Poly (A) $^+$ RNA was denatured in 6.5% formaldehyde/50% formamide at 65°C, size-fractionated on a 1.2% agarose gel containing 0.66% formaldehyde, and transferred to nitrocellulose membrane, as described by Maniatis et al. (19). After cross-linking by ultraviolet irradiation, these blots were analyzed using a full-length ^{32}P -cDNA probe of human heart chymase (14) under highly stringent washing conditions (0.1 \times SSC, 0.1% SDS, 65°C). To compare nonfailing and the idiopathic cardiomyopathic hearts, levels of the human heart chymase mRNA expression were normalized by the level of α -actin expression (cytoskeleton type) in the same blots.

Western blot analyses. The extraction of chymase from each tissue was performed as follows: A half gram of each tissue was homogenized in 20 mM Tris-HCl buffer, pH 7.4, and centrifuged at 40,000 g for 20 min. This procedure was repeated twice. The pellet was resuspended in 20 mM Tris-HCl buffer, pH 8.0, containing 2.0 M KCl and 1% Triton X-100, and incubated with gentle shaking at 4°C overnight. The mixture was centrifuged as above, and the resultant supernatant was incubated with 50 μ l of soybean trypsin inhibitor immobilized-agarose suspension (Pierce Chemical Co., Rockford, IL). After extensive washing of the soybean trypsin inhibitor-agarose gel with 20 mM Tris-HCl

buffer, pH 8.0, containing 2 M KCl and the same buffer without KCl, chymase bound to the gel was solubilized in 60 μ l of SDS-PAGE sample loading buffer at 22°C overnight. The protein solubilized in SDS-PAGE loading buffer and pure human heart chymase (100 and 200 ng standard) (13) were electrophoresed on 14% SDS-polyacrylamide gels, and the proteins were then transferred to a polyvinylidene difluoride microporous membrane using SEMI-PHOR TE 70 purchased at Hoefer Scientific Instruments (San Francisco, CA). The membranes containing the electroblotted proteins were analyzed by Western blot analyses using 125 I protein A, as previously described (13).

Enzymatic assay for chymase-like activity in human tissues. A half gram of each tissue was homogenized as described above, and the pellet was resuspended in 2 ml of 20 mM Tris-HCl buffer, pH 8.0. 5 μ l samples, preincubated for 30 min at room temperature with 1 mM EDTA, 1 mM *o*-phenanthroline, 20 μ M aprotinin, and with or without 10 μ M chymostatin, were incubated for 60 min at 37°C with 10 nmol Ang I in 50 μ l of 20 mM Tris-HCl buffer, pH 8.0, containing 0.5 M KCl and 0.01% Triton X-100. Generated Ang II was analyzed using a C₁₈ reverse-phase HPLC column (Vydac, Hesperia, CA) (13, 15). The peak area corresponding to a synthetic Ang II standard was integrated to calculate Ang II formation. Chymostatin-inhibitable Ang II formation was considered to represent the chymase-like activity and was expressed as nanomoles of Ang II formed/minute per gram tissue (wet weight). Other known Ang II-forming enzymes including ACE, cathepsin G, kallikrein, chymotrypsin, trypsin, and carboxypeptidases were completely inhibited in this assay procedure (data are not shown).

Statistical analyses. Autoradiographic data were analyzed using a densitometric image analyzer (densitometer) driven by a MacIntosh computer. To normalize the peak area, the value of the area of interest was divided by the corresponding value of the denominator. In Northern blot analyses of nonfailing and idiopathic cardiomyopathic hearts, α -actin was used as a denominator, and, in Western blot analyses, standardized amounts (100 and 200 ng) of the purified human heart chymase were used as a denominator. In analyzing the arbitrary unit value for the two transcripts resulting from Northern blot analyses, one-way ANOVA and Wilcoxon rank-sum tests were used for comparing nonfailing and idiopathic cardiomyopathic hearts. In analyzing chymase-like immunoreactivity and activity in the four chambers, a paired *t* test was used for comparing the chambers. In addition to this analysis, a mixed model analysis of variance was used with heart as a random effect and the fixed effects representing the chamber type (ventricle vs atrium) and side (left vs right). In the analyses of chymase-like immunoreactivity in the middle region of the left ventricular free wall among nonfailing, ischemic, and idiopathic cardiomyopathic hearts, one-way ANOVA, and Kruskal-Wallis tests were used for making the comparisons among the groups. *P* < 0.05 was considered statistically significant.

Results

EM immunocytochemical localization of chymase in the human heart. To examine the cellular localization of chymase in the human heart, EM immunocytochemical studies with a specific polyclonal antibody for human heart chymase were performed. Fig. 1 shows the localization of 10-nm immuno-gold deposits on some but not all secretory granules of a cardiac mast cell. The degree of immuno-gold labeling in these mast cell granules was generally low. Mast cell granules that contained the immuno-gold deposits had a dense core structure surrounded by a translucent area with a lattice structure similar to that of the tryptase/chymase (TC)-type mast cell (20, 21). EM-immunocytochemical studies, such as those of Craig et al. (21), have also shown that human chymase is stored in mast cell granules but that the degree of labeling of the TC type mast cell granules with a human chymase antibody is relatively weak and in some cases absent. The low degree of labeling could be

caused by a tight packing within granules of chymase, heparin, and other granule components (22). This type of packing may limit antigen presentation. As is apparent in Fig. 1, once released, contents of the mast cell granules show a much higher degree of labeling for chymase, which may occur because of an improvement in antigen presentation.

Immuno-gold deposits were also observed in cytosolic granules of endothelial cells located in intramural capillary. An example of this is shown in Fig. 2. The immuno-gold labeled cytosolic granules in several endothelial cells contained a microtubular structure, which is a typical ultrastructure of Weibel-Palade body containing von Willebrand factor (23). Cytosolic granules found in some mesenchymal cells of the left ventricular interstitium were also labeled with the immuno-gold deposits (Fig. 3). These mesenchymal cells were enriched in endoplasmic reticulum and contained several vacuoles. These ultrastructural properties are consistent with the properties of actively dividing fibroblasts (24).

The majority of immuno-gold deposits was observed in the cardiac interstitial region (Fig. 4 A). The immuno-gold deposits appeared to be associated with the extracellular matrix. No significant immuno-gold deposits were observed in myocytes or on the sarcolemmal membrane of any of the sections examined (Fig. 4 A). A low level of immuno-gold deposits, diffuse in pattern, was observed when preimmune serum was used in place of antisera against human heart chymase (Fig. 4 B); this type of labeling is characteristic of nonspecific binding.

Localization of chymase mRNA in the heart by *in situ* hybridization. To examine whether the immuno-gold deposits appearing in interstitial cell cytosolic granules are caused by chymase synthesis in interstitial cells or to protein endocytosis, *in situ* hybridization studies were carried out using a specific human chymase oligonucleotide probe. Dark and bright field views of a right ventricular section shows that chymase mRNA in the heart is localized in interstitial cells, as well as in endothelial cells of intramural arterioles (Fig. 5). An adjacent section stained by alcian blue for the identification of mast cells shows that several mast cells, but not the number equivalent to the number of chymase mRNA positive cells in Fig. 5 B, appeared in the media and adventitia region of the same intramural arteriole. Fig. 6 shows bright and dark field views of a section stained by hematoxylin and eosin, showing that interstitial and endothelial cells in intramural venula also contain chymase mRNA. An adjacent section produced by the radiolabeled sense probe shows low level and diffuse distribution of grains characteristic of nonspecific binding (Fig. 6 C).

Southern blot analyses of human genomic DNA. Southern blot analyses were performed using seven different restriction enzymes. One distinct hybridizing species is apparent in the EcoR1, EcoR1 + Xba1, Rsa1, and Pst1 digests; two in the Pvu2 and the Hind3 digests; and three in Sac1 digest (Fig. 7). The human chymase gene contains one Pvu2 site; two Sac1 and Hind3 sites; three Pst1 sites but no EcoR1, Xba1, and Rsa1 sites (14, 25). Since one Hind3 and two Pst1 fragments in the human chymase gene contain short encoding regions (76 bp, 50 bp, and 140 bp, respectively), these three fragments were not capable of being visualized in this Southern blot analysis. In all six different digests, however, there were no additional bands from the number expected as a result of the restriction map of the human chymase gene (14). These results suggest that, unlike rodent chymases (26–28), human chymase is likely to be a single gene product.

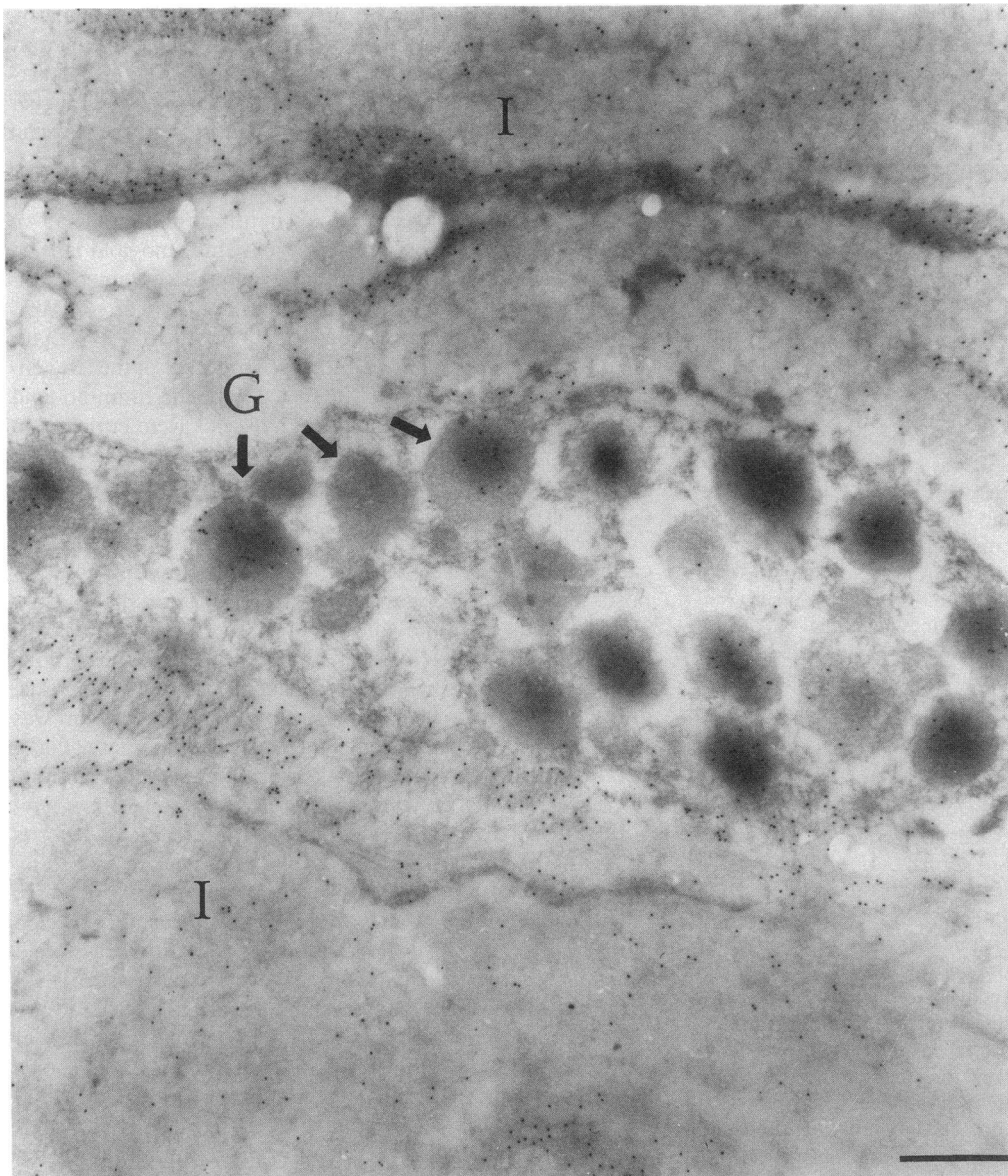


Figure 1. Immunohistochemistry of human left ventricular tissue using antichymase antibody. This tissue sample was from a donor heart of a 26-yr-old male. Immuno-gold electron microscopy identifies chymase-like immunoreactivity to be localized in mast cell granules (*G*, arrows). The cytosolic region of this mast cell is filled by large secretory granules that appear to have a dense core structure surrounded by a translucent area with a lattice structure. Immuno-gold deposits are also localized in the interstitial area (*I*). Bar, 500 nm.

Regional distribution of chymase mRNA, chymase-like immunoreactivity, and activity in the human heart. An example of Northern blot analyses of chymase mRNA in atria and ventricles from a male donor is shown in Fig. 8. Two transcripts (~ 1.4 and ~ 0.9 kb) were identified for each chamber of the heart and are likely caused by differential polyadenylation for the following reasons: (a) these two bands were equally intense even under highly stringent washing conditions; (b) the homology at the nucleotide level of the closest member to human heart chymase (e.g., cathepsin G or cytotoxic cell protease) is $\leq 50\%$ (14); (c) human chymase is likely a single gene product (see previous section); (d) only a single 769-bp polymerase chain reaction product was obtained from the mRNA isolated from the nonfailing or failing hearts (14); and (e) a single chymase was purified to homogeneity from human heart tissue (13). However, an alternate promoter for the transcription or an alternate splicing of the nascent transcript is possible. No significant difference in the levels of these two chymase mRNA transcripts was found between the nonfailing ($n = 8$) and the

failing idiopathic cardiomyopathic hearts ($n = 10$) (70 ± 10 vs 51 ± 6 arbitrary densitometric units for 1.4-kb bands, $P = 0.12$; 38 ± 13 vs 34 ± 5 arbitrary densitometric units for 0.9-kb bands, $P = 0.74$, respectively).

Northern blot analyses indicate that the ratio of the chymase mRNA to poly (A)⁺ RNA in each cardiac chamber is not remarkably different (Fig. 8). Because there could be differences in the efficiency of mRNA extraction between regions of the heart, it is uncertain if the levels of chymase mRNA among cardiac chambers are similar or are different.

Regional differences in chymase-like immunoreactivity and activity in each chamber of five nonfailing donor hearts were estimated by Western blot analyses (Figs. 9 and 10 *A*) and enzymatic activity assays (Fig. 10 *B*). A significant correlation was observed between chymase-like immunoreactivity and activity in nonfailing donor heart tissue ($y = 7.8x + 20$, $r^2 = 0.54$, $n = 18$, $P < 0.001$) (Fig. 10 *C*). When analyses were done according to chamber type (ventricles, $n = 10$, vs atria, $n = 8$), both chymase-like immunoreactivity ($P < 0.02$) and activity

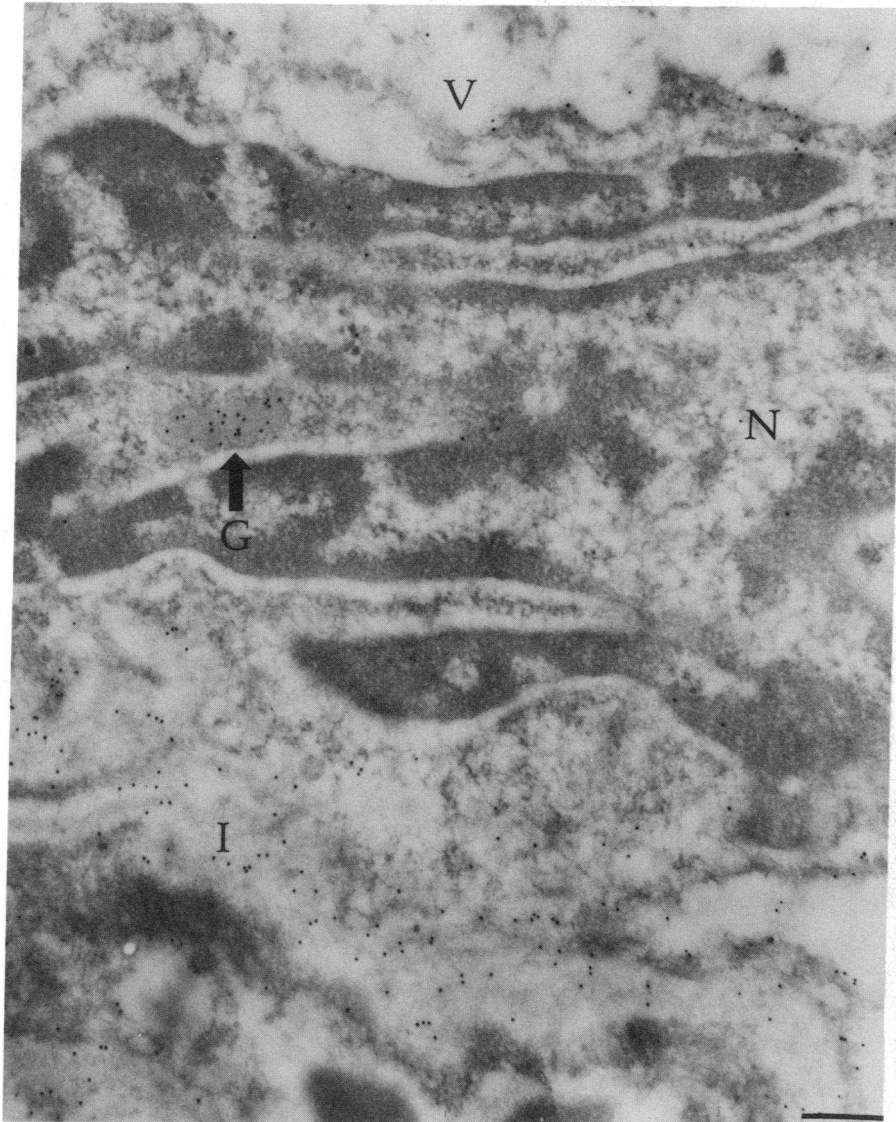


Figure 2. Immunohistochemistry of an endothelial cell from a cardiac intramural capillary using antichymase antibody. This tissue sample was from a donor heart of a 26-yr-old male. Immuno-gold electron microscopy identifies chymase-like immunoreactivity to be localized in endothelial cell granules (*G*, arrow). This granule has an internal microtubular structure, which is typical of Weibel-Palade body. Immuno-gold deposits are also found in the interstitial region (*I*) surrounding this endothelial cell. *N*, nucleus of endothelial cell; *V*, capillary lumen. Bar, 350 nm.

($P < 0.05$) were approximately twofold higher in ventricles than in atria (chymase-like immunoreactivity, ventricles 14 ± 1.4 vs atria 6.9 ± 1.1 arbitrary densitometric units, respectively; chymase-like activity, ventricles 140 ± 13 vs atria 61 ± 12 nmol Ang II formed/min per g tissue, respectively). When analyses were done according to side of the heart (right, $n = 10$, vs left, $n = 8$), there was no significant difference in chymase-like immunoreactivity ($P = 0.98$) and activity ($P = 0.41$) between the right and the left heart (chymase-like immunoreactivity, right 10 ± 1.5 vs left 11 ± 2.0 arbitrary densitometric units, respectively; chymase-like activity, right 105 ± 21 vs left 102 ± 14 nmol Ang II formed/min per g tissue, respectively).

A summary of chymase-like immunoreactivity and activity in nonfailing human left ventricular tissue and in left ventricular tissue from patients with ischemic or idiopathic cardiomyopathy is presented in Table I. There was no significant difference in chymase-like immunoreactivity ($P = 0.45$) and activity ($P = 0.45$) among nonfailing, failing ischemic, and idiopathic cardiomyopathy hearts.

A summary of chymase-like immunoreactivity and activity in several human tissue is presented in Table II. Levels of chy-

mase-like immunoreactivity and activity in the cardiac ventricles, lung, and liver were generally higher than those of observed in atria, coronary artery, aorta, kidney, and spleen. In the skin, high level of chymase-like activity was observed, but levels of chymase-like immunoreactivity were low. It is, at present, unclear whether skin tissue contains high levels of an Ang II-forming enzyme that is immunologically dissimilar to chymase.

Discussion

Our recent studies with human heart tissue suggest the presence of a dual pathway of Ang II formation in which the serine proteinase chymase is the major and ACE is the minor Ang II-forming activity (12). Chymase isolated from the human heart has a high catalytic efficiency and specificity for the conversion of Ang I to Ang II, and has been proposed to play a significant role in the paracrine regulation of heart function by Ang II (12, 13). In this report, we describe the cells in the human heart that are involved in the synthesis of chymase and the localization of this enzyme once it is released. These obser-

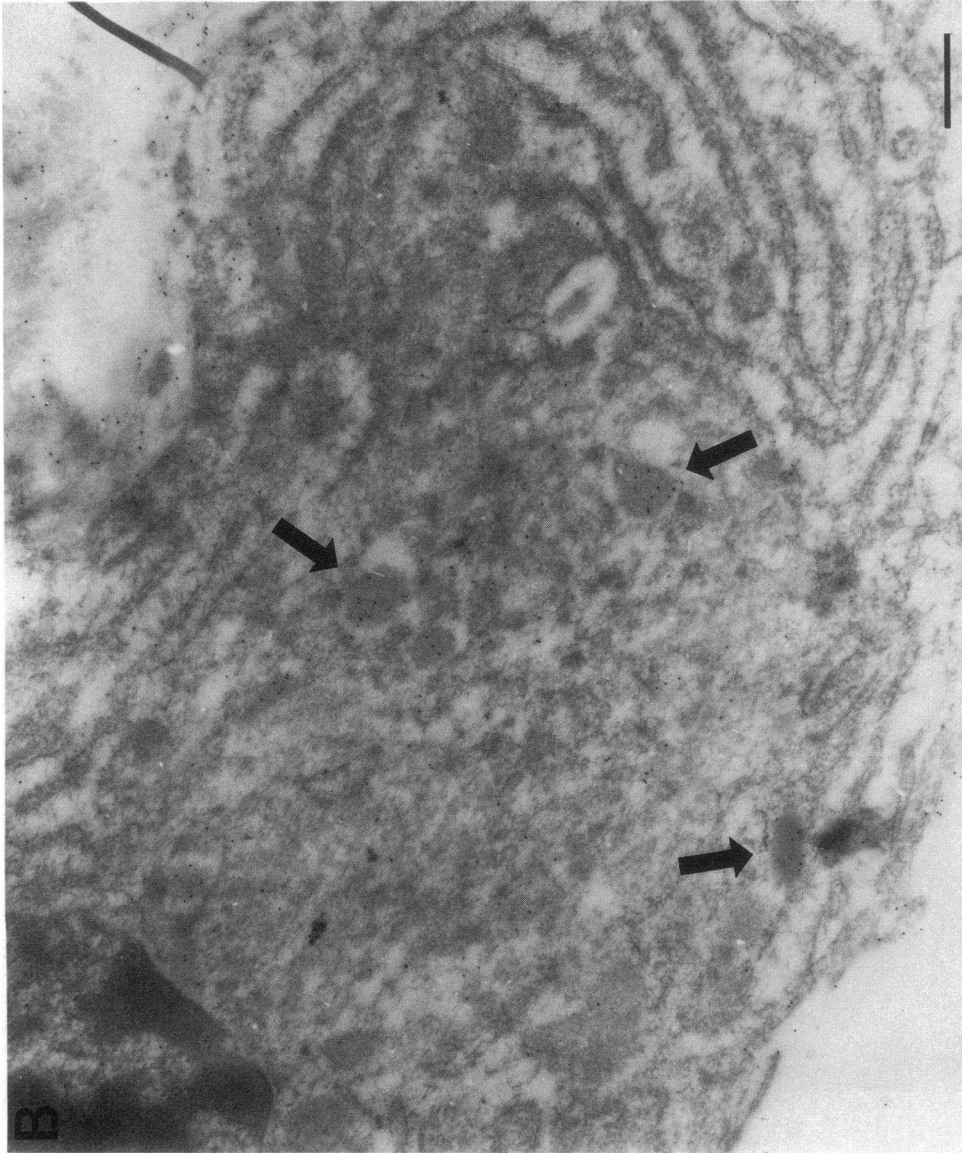
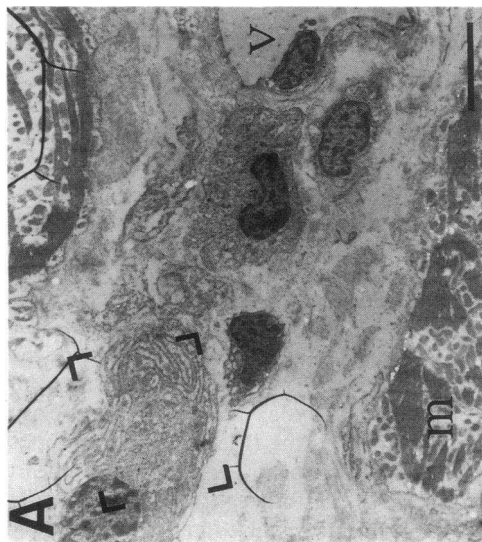


Figure 3. Immunohistochemistry of an interstitial cell in perivascular region of a human left ventricular tissue using antichymase antibody. This tissue sample was from a donor heart of a 26-yr-old male. (A) Low magnification view of a cardiac perivascular region. Solid bar, 2 μ m. *m*, myocyte; *V*, vascular lumen. A magnification of the bracketed area over a mesenchymal interstitial cell in this section is shown in B. (B) Immuno-gold deposits identifies chymase-like immunoreactivity in several cytosolic granules of this interstitial cell (arrows). This interstitial cell contains a prominent endoplasmic reticulum and several vacuoles, consistent with the ultrastructure of an activated fibroblast. Solid bar, 500 nm.

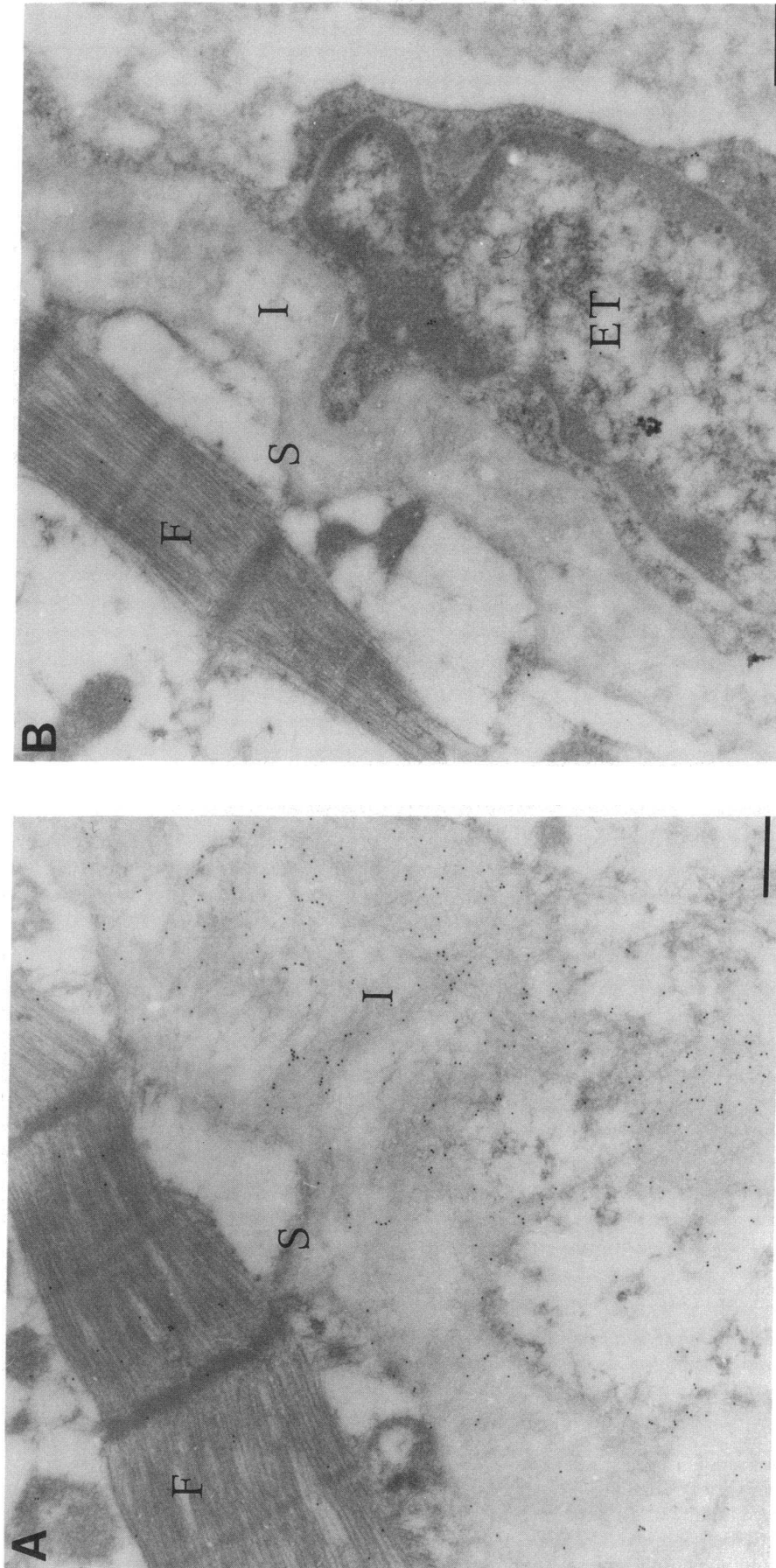


Figure 4 Immunohistochemistry of left ventricular myocardium from a nonfailing donor heart using antichymase antibody (A) and preimmune serum (B). (A) Immuno-gold deposits identifies chymase-like immunoreactivity in the interstitial region (I) of the cardiac ventricle, but not in myocytes and the sarcolemma (S). (B) Using preimmune serum, immuno-gold deposits were diffuse and their levels were low. This type of binding is characteristic of nonspecific binding. F, contractile myofibrilament; ET, endothelial cell. Bar, 200 nm.

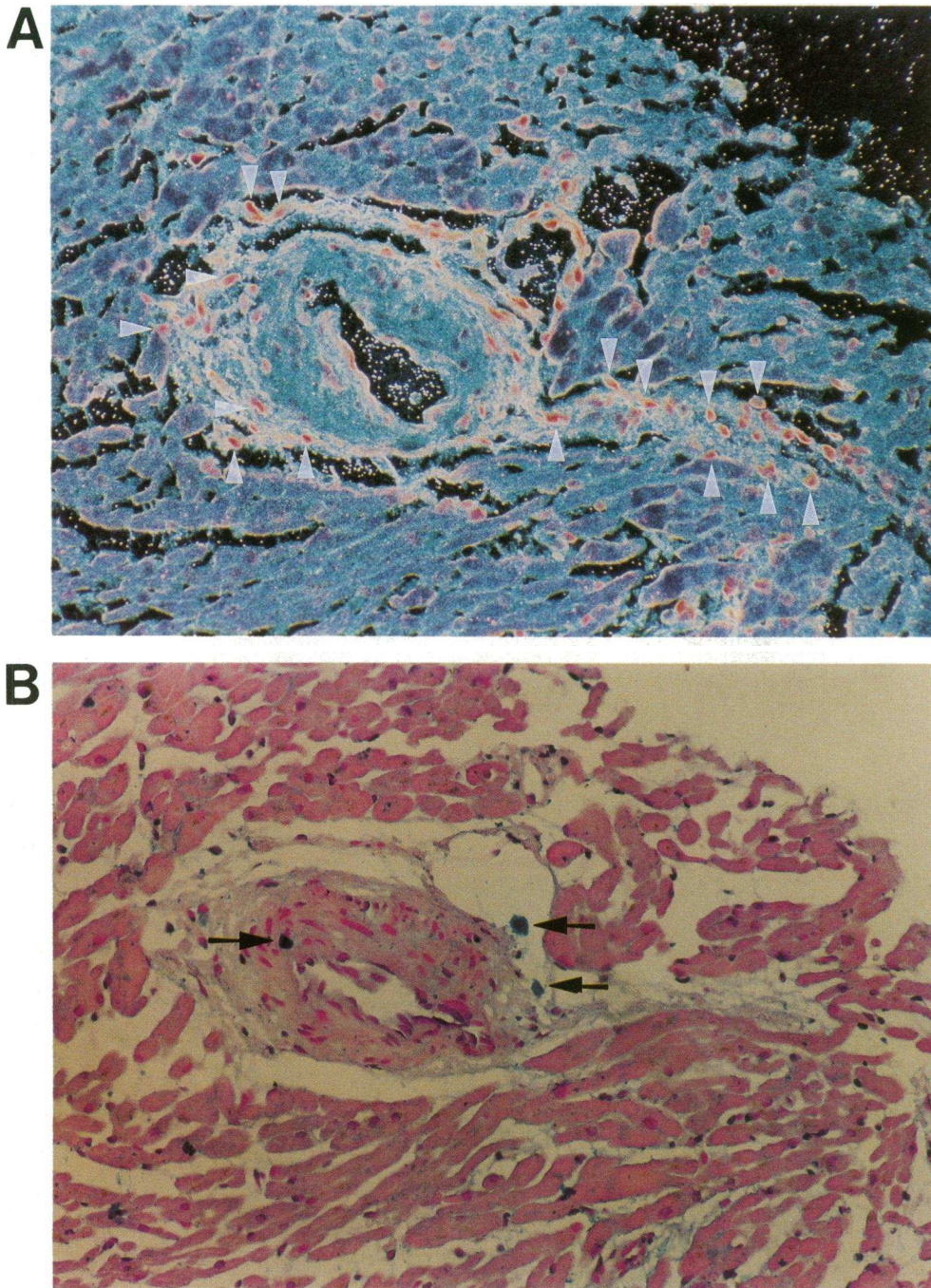


Figure 5. (A) Dark field view of a histological section of the right ventricle from a donor heart of a 26-yr-old male hybridized with a human chymase-antisense oligonucleotide probe. Bright spots represent human chymase mRNA. Several adventitial interstitial cells containing human chymase mRNA are indicated (*white arrowheads*). (B) An alcian blue stained histological section, adjacent to that shown in (A), indicates mast cells (*black arrows*). $\times 200$.

variations, as well as a systematic study of the regional distribution of chymase in the human heart, provide valuable insights into the role of chymase in cardiac Ang II formation.

EM-immunocytochemical studies using a specific human heart chymase polyclonal antibody indicate the presence of chymase-like immunoreactivity in secretory granules of human cardiac mast cells. This finding was expected, since all other mammalian chymases studied have been shown to be stored in an active form in secretory granules of the mast cell (29, 30). A highly unexpected finding was the observation that chymase-like immunoreactivity is present in Weibel-Palade bodies of endothelial cells and in cytosolic granules of intersti-

tial mesenchymal cells. These interstitial cells appeared to be actively dividing fibroblasts because of the presence of an extensive network of rough endoplasmic reticulum and cytoplasmic vacuoles (24). The human heart chymase antibody used in these subcellular localization studies did not cross-hybridize to other known serine proteinases closely related to chymase such as human neutrophil cathepsin G (13), suggesting that the vesicular chymase-like immunoreactivity was caused by chymase. To examine if unknown chymase-like proteinases highly homologous to human chymase may contribute to chymase-like immunoreactivity, Southern blot analyses of human genomic DNA were carried out using a cDNA probe compris-

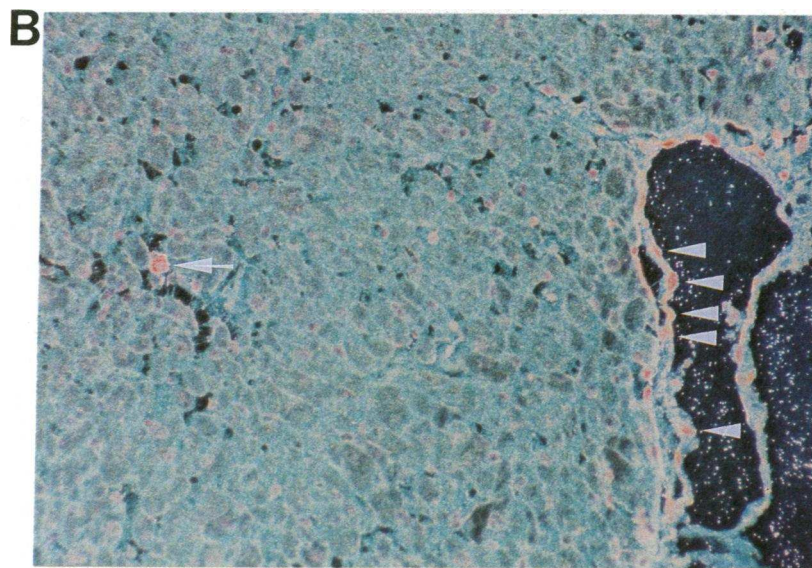
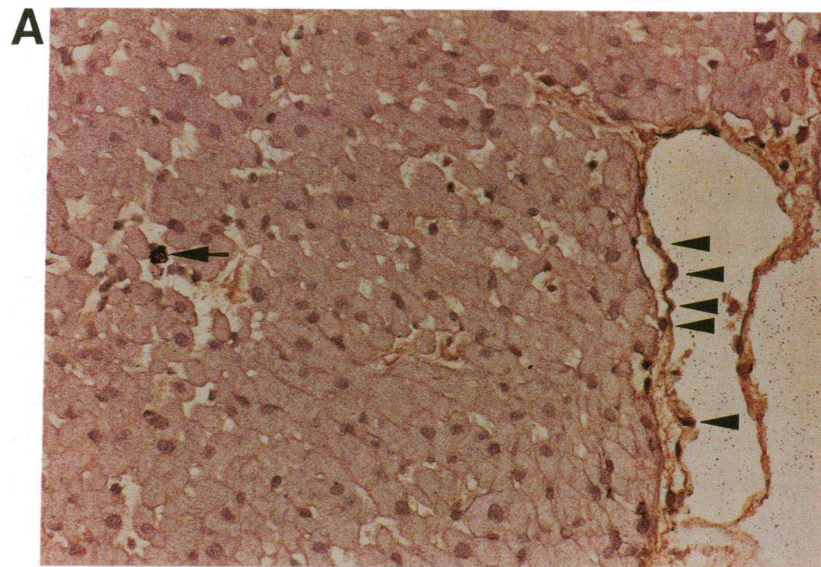


Figure 6. (A) Bright field and (B) dark field views of a histological section of the right ventricle from a donor heart of a 26-yr-old male hybridized with a human chymase-antisense oligonucleotide probe. Silver grains in A or bright spots in B represent human chymase mRNA transcripts. Based on alcian blue staining of an adjacent section (not shown), the heavily labeled interstitial cell indicated by an arrow appears to be a mast cell. A high density of silver grains are also found in endothelial cells (arrowheads). (C) Dark field view of a histological section, adjacent to that shown in A, hybridized with a sense probe complimentary to the probe used in A and B. The staining of this tissue section by hematoxylin & eosin was much weaker than that in B. Weak staining was necessary to show in a dark field view that the brightly lit silver grains in this control section had a diffuse pattern indicative of non-specific binding. $\times 170$.

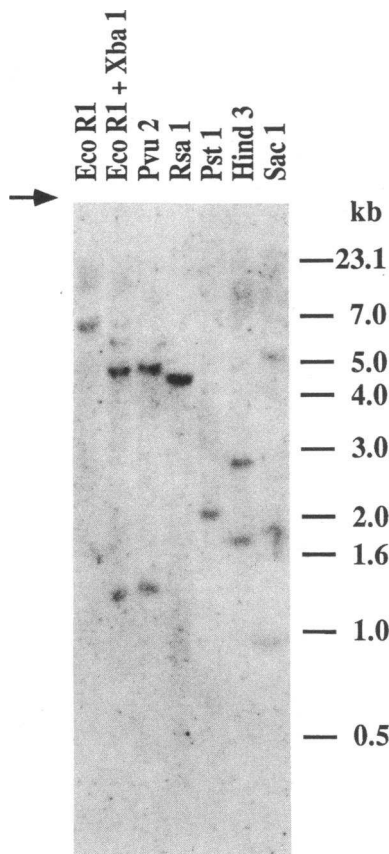


Figure 7. Southern blot analyses of human genomic DNA with human heart chymase cDNA probe. 7 μ g of human DNA were digested with restriction enzymes, as listed on the top of the figure. Fragments that hybridized the full-length 32 P-labeled human heart chymase cDNA probe were identified by Southern blot analyses. The analyses was performed under low stringent washing conditions, as described. One distinct hybridizing species is apparent in the EcoRI, EcoRI + XbaI, RsaI, and PstI digests; two in the Pvu2 and Hind3 digests; and three in the SacI digest. The human heart chymase gene contains one Pvu2 site, two SacI and Hind3 sites, three PstI sites, but no EcoRI, XbaI, and RsaI sites. Since one of the Hind3

and two of PstI fragments contain the short encoding region (76, 50, and 140 bp, respectively) of the human heart chymase gene, these three fragments were not capable of being visualized in this Southern blot analyses. Please note that, in any of six different digests including RsaI, no additional bands other than those expected from the restriction map of the human heart chymase gene were found, suggesting that there is a single gene for human chymase. This Southern blot is representative of three different blots obtained using genomic DNA from three humans.

ing the entire coding region of human heart preprochymase. Extensive low stringency Southern blot analyses indicated that human chymase is a single gene product, supporting the contention that the human heart chymase antibody did not cross-react with unknown proteins highly homologous to human chymase. These observations indicate that the chymase-like immunoreactivity detected in cytosolic granules of mast cells, endothelial cells, and some interstitial cells is likely to be caused by chymase.

Based on their ultrastructural characteristics, cytosolic vesicles containing chymase-like immunoreactivity in mast cells and endothelial cells, but not those of interstitial cells, appeared to be secretory (31, 32). To clarify whether vesicular chymase-like immunoreactivity in interstitial cells is a product of cellular biosynthesis or occurred through protein uptake, in situ hybridization studies were carried out. Using a highly specific antisense oligonucleotide probe, chymase mRNA signal was observed in endothelial cells of the intramural venula and in several interstitial cells including mast cells. These in situ hybridization studies, as well as the EM-immunocytochemical studies, are the first to show that human chymase is elaborated

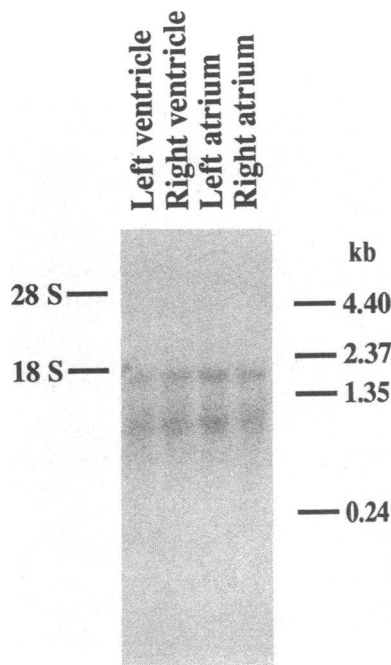


Figure 8. A representative Northern blot of chymase mRNA expression in the human heart. Poly (A)⁺ RNA was isolated from one gram of a nonfailing donor heart and 3 μ g of poly (A)⁺ RNA was used for Northern blot analyses, as described. Exposure time for development was 7 d. Two transcripts (\sim 1.4 and \sim 0.9 kb) were observed in each chamber of the heart.

in cell types other than the mast cell. This is a unique finding, since other known chymases have been described only in mast cells (29, 30, 33). It is interesting to note that the 5' and 3' untranslated region of human chymase gene lacks important mast cell specific enhancer sequences found in mouse and rat chymase genes (14, 25, 34). It is tempting to suggest that such differences in the *cis*-acting elements of the human chymase gene may account for its more widespread cellular distribution than other mammalian chymases. Because chymases previously have only been identified in mast cells, they have often been referred to as mast cell proteases; e.g., rat mast cell protease I, rat mast cell protease II (29, 30). Because chymase is present in multiple cell types in the human heart, we believe

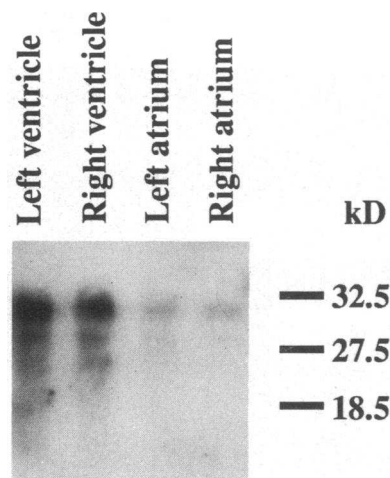


Figure 9. A representative Western blot showing chymase-like immunoreactivity in the human heart. A half gram of tissue from each chamber of a donor heart was analyzed for chymase-like immunoreactivity, as described in Methods. A major immunoreactive band, \sim 30 kD, was observed in each heart chamber, however, lower molecular weight immunoreactive bands of lesser intensity were also observed. We have

previously shown that human chymase remains active after proteolytic cuts are made in noncritical regions of the enzyme (13). However once the enzyme is cut, it appears as a lower molecular weight band when analyzed by SDS-PAGE under reducing conditions (13).

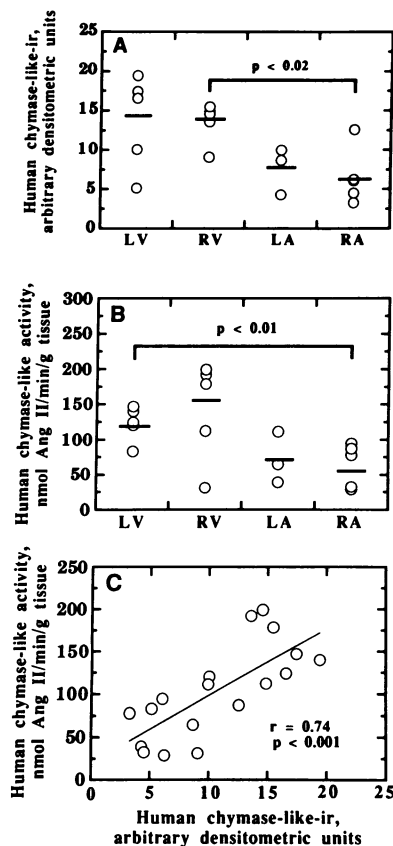


Figure 10. Regional distribution of chymase-like immunoreactivity and activity in the human heart. Chymase-like immunoreactivity (*ir*) (A) and activity (B) were determined in each chamber of five nonfailing hearts (two left atrial tissue could not be obtained), as described in the Methods section. Based on control denominators (100 or 200 ng pure human heart chymase), autoradiographic data in Western blots at 30 kD (size of native human chymase in the heart) were converted to the arbitrary densitometric units. Horizontal bars represent mean values. (C) A significant correlation was observed between chymase-like immunoreactivity and activity ($y = 7.8x + 20$, $r^2 = 0.54$, $n = 18$). LV, left ventricle; RV, right ventricle; LA, left atrium; RA, right atrium.

that the name chymase is more appropriate than the name human mast cell protease, proposed by Jenne et al. (35). The name "chymase" is not limiting as the name "mast cell protease."

Using EM-immunocytochemistry, Kaminer et al. (36) have shown that chymase in isolated human skin mast cells is released in the extracellular space 1 h after a 2,4-dinitrochlorobenzene challenge; released chymase was observed attached to the extracellular matrix. High levels of chymase-like immunoreactivity are localized in the cardiac interstitium and are likely associated with the interstitial extracellular matrix. Human chymase is a highly basic enzyme (14). Molecular modeling studies on human chymase (Sung S. S., and A. Husain, unpublished observations) indicate the presence of several positively

Table I. Chymase-like Immunoreactivity and Enzymatic Activity in Left Midventricular Regions of Nonfailing and Failing Human Hearts

Pathological diagnosis	Chymase-like immunoreactivity	Chymase-like activity
	arbitrary densitometric units	nmol Ang II formed/min/g tissue wet wt
Nonfailing ($n = 5$)	64 ± 14	105 ± 11
Ischemic cardiomyopathy ($n = 5$)	36 ± 11	88 ± 15
Idiopathic cardiomyopathy ($n = 5$)	54 ± 20	100 ± 12

Table II. Chymase-like Immunoreactivity and Enzymatic Activity in Human Tissues

Tissues	Chymase-like immunoreactivity*	Chymase-like activity†
Left ventricle	+++	+++
Right ventricle	+++	+++
Lung	++	+++
Liver	++	++
Left atrium	+	++
Right atrium	+	+
Coronary artery	+	++
Aorta	+	+
Skin	+	+++
Kidney cortex	+	++
Kidney medulla	+	+
Spleen	ND	+

* +, < 7.5; ++, 7.5 to 15; and +++, > 15 arbitrary densitometric units. † +, < 30; ++, 30 to 70; and +++, > 70 nmol Ang II formed/min per g tissue (wet wt). ND, not detectable.

charged residues on the surface of this enzyme. In rat chymase I, these positively charged residues have been suggested to play a role in binding to heparin or to other sulfated proteoglycans and glycosaminoglycans found in secretory granules, and to the extracellular matrix (37). The highly basic nature of human chymase is common to all known proteinases found in mast cell granules including tryptase (38), carboxypeptidase A (39) and cathepsin G (40). It is also known that when these proteinases are bound to heparin or to heparan sulfate, they are relatively resistant to proteolytic degradation and to inactivation by the plasma serine proteinase inhibitor (41). Chymase in the heart may thus be relatively stable and likely remains active after binding to the extracellular matrix. This latter conclusion is supported by the fact that human chymase is active after binding to heparin immobilized on agarose (Urata, H., and A. Husain, unpublished observation). Ang II formation occurring from chymase activity associated with the extracellular matrix in the cardiac interstitium may thus be a major site of local Ang II formation in the human heart.

ACE inhibitors are widely used in the treatment of hypertension and congestive heart failure (2, 3, 42). Because it is presumed that Ang II levels in the failing heart are reduced substantially after ACE inhibitor therapy, some investigators believe that Ang II effects on the failing human heart are deleterious (6). The level of Ang II in human heart tissue is likely regulated by two mechanisms: (a) uptake of circulating Ang II; and (b) local conversion of Ang I to Ang II by ACE and potentially by chymase. In blood serum, ACE is the major enzyme that converts of Ang I to Ang II (12). By inhibiting ACE, Ang II formation in the circulation will be markedly reduced. Therefore, the fraction of Ang II in the heart that is taken up from the circulation also will be markedly reduced. Local Ang II formation in the heart caused by human cardiac ACE will also be reduced during ACE inhibitor therapy. On the other hand, since chronic ACE inhibitor therapy produces a > 300% increase in circulating Ang I levels (43), uptake of Ang I to the heart should be increased. Because chymase levels also are not reduced in cardiac ventricles of patients with idiopathic or isch-

emic cardiomyopathy, it is tempting to speculate that chymase-dependent conversion of Ang I to Ang II in heart tissue will be increased in patients undergoing ACE inhibitor therapy. We believe that the contribution of ACE and chymase to cardiac Ang II formation needs to be determined before conclusions are made about the role of cardiac Ang II in congestive heart failure. Such information may prove to be valuable in understanding the mechanism of action of ACE inhibitors and Ang II receptor antagonists in the treatment of hypertension and congestive heart failure, particularly if differences in efficacy are apparent between these drug classes.

During chronic ACE inhibitor therapy levels of circulating Ang II are only partially decreased (44). It has been suggested, but not proven, that even high doses of ACE inhibitors, produce incomplete inhibition of tissue ACE. It is conceivable, however, that during chronic ACE inhibitor therapy, Ang II formed by chymase in the heart and possibly in other tissues contributes to blood Ang II levels. To investigate the potential contribution of tissue chymase in regulating circulating Ang II levels, we determined the distribution of chymase in several human tissues. The presence of chymase-like immunoreactivity and activity in the skin, lungs, liver, and coronary arteries would suggest that tissues other than the heart may contribute to levels of circulating Ang II observed during chronic ACE inhibitor therapy. With respect to vascular chymase-like activity, it is interesting to note that Ang I-mediated contraction of monkey mesenteric and pulmonary arterial strips has been reported to be partially inhibited (by ~ 55%) by an ACE inhibitor, but completely inhibited by combined ACE inhibitor and chymostatin treatment (45). Chymostatin, a nonspecific inhibitor of chymotrypsin-like proteinases which inhibits human chymase, could inhibit Ang I-mediated vascular contraction by inhibiting chymase-like activity in monkey vessels.

Chymase-dependent Ang II formation may be greater in human cardiac ventricles than in atria since our study shows a twofold higher level of chymase-like immunoreactivity and activity in ventricles than in atria. ACE activity in the human heart also shows regional variations (12). Levels of ACE are approximately threefold higher in the right atrium than in the left ventricle and are twofold higher in the right ventricle than in the left. Under chronic ACE inhibitor therapy, differential changes in cardiac Ang II concentration may occur. Because of the relative distribution of ACE and chymase in the human heart, atrial Ang II levels may be reduced more significantly than left ventricular Ang II levels during chronic ACE inhibitor therapy. In addition to regional differences in levels of chymase and ACE, differences in the enzymatic properties of chymase and ACE may also be important. Chymase and ACE are highly efficient Ang II-forming enzymes. However, unlike ACE, chymase is more specific and does not cleave bradykinin and substance P (13, 15). This observation would suggest that chymase-dependent Ang II formation is not coupled to the simultaneous inactivation of vasodilator peptides as has been shown with ACE, a role perhaps more consistent with the regulation of myocyte function than with blood pressure regulation.

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