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Original Article

Expression of the Cysteine Proteinase Inhibitor Cystatin C Gene in Rat Heart: Use of Digoxigenin-labeled Probes Generated by Polymerase Chain Reaction Directly for In Situ and Northern Blot Hybridizations¹

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Cystatins represent a widely distributed superfamily of cysteine proteinase inhibitory proteins. We investigated the expression of the cystatin C gene, belonging to the family 2 of cystatins, in the hearts of female rats. Using a highly sensitive reverse transcriptase-polymerase chain reaction (RT-PCR) we have detected cystatin C mRNA in the ventricle and atrium, as well as in liver and submandibular gland. A digoxigenin-labeled cystatin C probe, generated by PCR, hybridized to a single mRNA species of about 700 nucleotides on Northern blots. Northern blot hybridizations established that neither an acute inflammation produced by injection of turpentine nor administration of the β -adrenergic

agonist isoproterenol had an effect on the level of cystatin C mRNA in the heart. In situ hybridizations with digoxigenin-labeled probe localized the expression of the cystatin C gene to cardiac muscle fibers but not to other cardiac cellular elements. Cystatin C may be released by cardiac muscle fibers under physiological and pathological conditions and may modify inflammatory and necrobiotic processes. (*J Histochem Cytochem* 41:1863-1867, 1993)

KEY WORDS: Polymerase chain reaction; In situ hybridization; Northern blot hybridization; Digoxigenin-labeled probe; Heart; Cystatin; Cysteine proteinase inhibitor.

Introduction

Cystatins, widespread in living organisms, are inhibitors of cysteine proteinases. All known cystatins belong to the cystatin superfamily consisting of stefins, cystatins, and kininogens (3,19). In preliminary experiments we have found that induction of cardiac hypertrophy by infusion of the β -adrenergic agonist isoproterenol led to an increase in the sum of cysteine proteinase inhibitory activity, measured by titration of papain inhibitory activity, of heart extracts. This observation led us to a systematic analysis of cardiac cystatins.

Cysteine proteinase inhibitors have been described in the hearts of several species (9,10,15,18,20) but, to our knowledge, the expressions of cystatin genes in cardiac tissues have not been documented. Using a highly sensitive reverse transcriptase-polymerase chain reaction (RT-PCR) and in situ and Northern blot hybridizations employing digoxigenin-labeled probes, we show that the cystatin C gene is expressed in cardiac muscle fibers. Furthermore, we docu-

ment that the level of cystatin C mRNA is not altered by acute inflammation or by β -adrenoreceptors.

Materials and Methods

Animals and Treatments

Adult female Sprague-Dawley rats weighing 160-180 g were kept in air-conditioned quarters and had access to standard laboratory chow. Acute inflammation was induced by SC injection of 0.5 ml/100 g body weight (b.w.) of turpentine in two sites. The animals were deprived of food and were sacrificed 24 or 48 hr after injection of the turpentine. Control rats were fasting for the same lengths of time. Rats were injected IP with 0.1 μ mol/g b.w. of isoproterenol-HCl (IPR) (Sigma; St Louis, MO). The IPR was dissolved in 0.1% sodium metabisulfite in PBS. The hearts were removed 1, 4, 18, or 24 hr after injection of IPR. Chronically treated rats received six daily injections of IPR and the heart was removed 24 hr after the last injection of the drug. Some rats were treated with IPR for 6 days and the hearts were removed after a 6-day recovery period. During these experiments the Institution's guide for care and use of animals was followed.

Reverse Transcriptase-Polymerase Chain Reaction

For detection and analyses of the expression of cystatin C gene at the mRNA

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level we used reverse transcription of mRNAs and PCR amplification of cDNAs as well as Northern blot hybridization. The reverse transcription of RNA to cDNA and the subsequent amplification were carried out using, for both reactions, the thermostable *flfh* DNA polymerase according to the instructions of the manufacturer (GeneAmp Thermostable *flfh* Reverse Transcriptase RNA PCR Kit, Part No. N808-0069; Perkin Elmer Cetus, Norwalk, CT).

For the PCR amplification of cystatin C cDNA the primers were: sense primer, CYSC-A, 5'-GTAAGCAGCTTGCTGGAA-3' (NT positions 181–201 of rat cystatin C cDNA); anti-sense primer, 5'-GCCTTCTTACTGTC-TCCCTGGT-3' (NT positions 500–520 of rat cystatin C cDNA) (GenBank, LOCUS: RATCYSC, ACCESSION: X16957) (7). Using these primers, a 339 bp fragment (including primers) of cystatin C cDNA was amplified.

The reaction volume for PCR was 100 μ l. The concentrations of primers were 0.5 μ M each and those of the deoxynucleotides 200 μ M. The RT reaction mixture contained 250 ng of total RNA and the reaction was for 15 min at 70°C. After a 2-min denaturation at 95°C, each of the 35 or 40 PCR cycles consisted of denaturation at 95°C, annealing at 55°C, and extension at 72°C, each for 1 min. This was followed by a 7-min elongation at 60°C. DNA was electrophoresed in 2% agarose gel (Perkin-Elmer; Catalog No. 61408JB) and visualized by ethidium bromide staining.

Northern Blot Analysis

RNA was isolated by the guanidine isothiocyanate–cesium chloride gradient method (6). It was precipitated twice with ethanol and dissolved in RNase-free 1 mM EDTA, 10 mM NaCl, 10 mM Tris-HCl, pH 8. The RNA was used for both Northern blot hybridizations and RT-PCR.

Total RNA, 15 μ g/lane, was electrophoresed through 1% agarose gel containing 6% formaldehyde, blotted onto positively charged nylon membranes (Boehringer Mannheim; Indianapolis, IN), and cross-linked for 3 min with UV Stratalinker 1800 (Stratagene; La Jolla, CA). Before blotting the ethidium bromide-stained RNA was visualized and photographed. For Northern blot hybridization, digoxigenin-labeled DNA probe was used. Hybridization and detection were carried out according to the instructions of the manufacturer (the Genius System; Boehringer Mannheim) as follows: pre-hybridization for 2 hr and hybridization overnight, both at 55°C. The pre-hybridization solution consisted of 5 \times SSC (1 \times SSC = 0.15 M NaCl, 0.015 M trisodium citrate), 50% formamide, 0.02% sodium dodecyl sulfate (SDS), 0.1% *N*-lauroylsarcosine, and 2% blocking reagent. The hybridization solution was the same as the pre-hybridization solution containing about 20 ng/ml of the digoxigenin-labeled DNA probe. After hybridization the membrane was washed twice for 5 min in 2 \times SSC, 0.1% SDS at room temperature, and then twice for 15 min in 0.5 \times SSC, 0.1% SDS at 65°C. Detection of the hybridized probe was with anti-DIG-alkaline phosphatase antibody. Incubation with the antibody (1:5000 dilution) was for 60 min at room temperature. Alkaline phosphatase activity was demonstrated by incubation with the substrate 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitroblue tetrazolium (NBT) for 2–18 hr at room temperature in the dark.

Preparation of the Digoxigenin-labeled Cystatin Probe

The digoxigenin-labeled probe was generated in a PCR using AmpliTaq DNA polymerase (GeneAmp PCR Core Reagents; Perkin Elmer Cetus) in three steps: (a) RT-PCR using the CysC-A and CysC-B primers and total liver RNA as template, as described above; (b) a second amplification using 1 μ l of the reaction mixture from the RT-PCR reaction as template directly; (c) amplification using digoxigenin-11-dUTP and 1 μ l of the reaction mixture from the second amplification as template directly. In preliminary experiments we had established that aliquots of the RT-PCR or PCR reaction

mixture can be used for subsequent amplification without purification of the amplified fragments separated by agarose gel electrophoresis. The reaction mixture (100 μ l) (13) contained dATP, dCTP, dGTP, each 200 μ M, dTTP 130 μ M, digoxigenin-11-dUTP 70 μ M, primers (CysC-A and CysC-B) 0.5 μ M each, AmpliTaq DNA polymerase 0.5 μ l (2.5 U/100 μ l), MgCl₂ 1 mM, template, 1 μ l of amplification mixture from Amplification b. The 40 PCR cycles were as described above. The approximate yield of DIG-labeled DNA was estimated using a DIG-labeled control DNA supplied by the manufacturer. The PCR reaction mixture was placed in boiling water for 10 min, and was stored at –20°C. A calculated aliquot (about 1 μ l/ml) was added directly to fresh pre-hybridization mixture. When reused, the hybridization mixture was heated to 65°C for 10 min.

In Situ Hybridization

Unless otherwise stated, the reagents used for the *in situ* hybridizations were obtained from Boehringer Mannheim and the protocols given by the manufacturer were followed with modifications described below. For all dilutions, diethyl pyrocarbonate-treated sterile water was used (1 ml diethyl pyrocarbonate/liter of water, overnight, autoclaved). All glassware was baked for 4–6 hr at 160°C.

Preparation of Slides for Hybridization. Tissues were fixed in 4% paraformaldehyde in PBS for 1.5–2 hr at room temperature. The sterile PBS was diluted from a 10 \times stock solution. For cryoprotection the tissues were transferred, without washing, into 15% sucrose (RNase free) (Schwarz/Mann; Orangeburg, NY) in sterile PBS and were kept overnight at 4°C. The tissues were quenched in liquid nitrogen-chilled isopentane and stored at –80°C. Cryostat sections 8 μ m thick were cut at –20°C and were mounted on silane-coated slides (Polysciences; Warrington, PA). The sections were encircled with a marking pen (PAP PEN; Jersey Lab Supply, Livingston, NJ), dried at room temperature for 1–2 hr, and stored at –80°C. Before pre-hybridization the sections were fixed in 4% paraformaldehyde in PBS for 5 min, washed twice for 5 min in sterile PBS and for 10 min in 2 \times SSC.

Pre-hybridization. Pre-hybridization was for 1–2 hr at 55°C. The pre-hybridization solution contained 50% formamide, 6 \times SSC, 5 \times Denhardt's solution (1 \times Denhardt's solution = 0.1 g polyvinylpyrrolidone, 0.1 g bovine serum albumin, 0.1 g Ficoll 400, H₂O to 500 ml), 0.1 mg/ml sperm DNA, 10% dextran sulfate, 5 mg/ml sodium pyrophosphate, and 0.5% SDS.

The pre-hybridization solution (1 ml) was prepared from stock solutions as follows: 0.5 ml formamide, 25 μ l 20% SDS, 10 μ l 0.5% sodium pyrophosphate, 0.465 ml 2 \times hybridization solution (Gibco; Grand Island, NY), 10 μ l of 0.1 M levamisol (final concentration 1 mM). Finally, 100 mg of dextran sulfate was added and the mixture was vortexed until the dextran was dissolved. Aliquots of the 2 \times hybridization solution and the levamisol stock solution were kept at –20°C.

Hybridization. The hybridization solution was the same as the pre-hybridization solution to which the DIG-labeled probe was added to a final concentration of about 1 μ g/ml (20 ng/20 μ l). Preliminary experiments established that an aliquot of the PCR reaction mixture can be used for hybridization (for both Northern and *in situ*) without purification of the labeled fragments. The probe was heated each time in boiling water for 10 min and chilled on ice. Each section was layered over with 20 μ l of the hybridization solution and was covered with a silane-coated coverslip. Hybridization was at 55°C (25°C below T_m) overnight in a wet chamber. The chamber was wetted with 2 \times SSC, 50% formamide.

Washings. Washings were twice for 15 min in 2 \times SSC at room temperature, four times for 15 min in 0.1 \times SSC at 55°C; 5 min in 0.1 \times SSC at room temperature, and 5 min in Buffer 1 (100 mM Tris-HCl, pH 7.5, 150 mM NaCl) at room temperature.

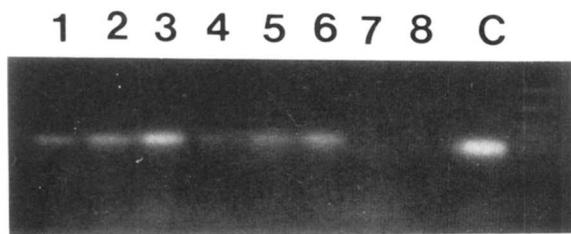


Figure 1. RT-PCR analysis of cystatin C expression in heart, liver, and submandibular gland of control and turpentine-injected rats. Agarose gel electrophoresis of PCR-amplified cystatin C cDNAs. Amplification of a 339 bp fragment is seen with RNAs from all three organs. In the liver and submandibular gland the acute inflammation seemed to reduce the level of cystatin C mRNA. However, the RT-PCR method, as applied, is not quantitative. Total RNA was extracted from the organs of an untreated female rat and from the organs of a rat that received 0.5 ml/100 g b.w. of turpentine 48 hr earlier. An RT-PCR method was used to amplify cystatin C cDNA as described in Materials and Methods. Lanes 1–4, control rat; Lanes 5–8, turpentine-injected rat. Lanes 1 and 5, ventricles; Lanes 2 and 6, atria; Lanes 3 and 7, livers; Lanes 4 and 8, submandibular glands; Lane C, positive control RNA. With the primers supplied by the manufacturer (Perkin Elmer Cetus) a 308 bp product is amplified from pAW109-derived cDNA.

Detection. Detection involved the following steps: (a) blocking in 2% goat serum in Buffer 1, 0.3% Triton X-100 for 30 min; (b) blocking in 2% blocking solution for 30 min. The 10 × blocking stock solution was diluted with Buffer 1; (c) incubation with anti-DIG antibody, 1:250, diluted in 2% blocking solution for 1–2 hr at room temperature; (d) rinsing and then washing three times for 5 min in Buffer 1; (e) treatment with Buffer 3 (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 50 mM MgCl₂) for 10 min; (f) incubation of the sections in a Coplin jar in the substrate solution (1 ml Buffer 3, 4.5 µl NBT solution, 3.5 µl BCIP solution) for 4–16 hr. If overnight incubation was required the staining solution was diluted 2:1 with Buffer 3. The incubation was terminated by washing in water and the sections were dehydrated and mounted in Permount. If desired, the sections can be counterstained (e.g., with methyl green or Nuclear Fast Red).

Controls. For the *in situ* hybridization two controls were employed: hybridization in a solution without the DIG-labeled probe, and treatment of the sections with ribonuclease A (Sigma) (0.1 mg/ml of 2 × SSC) for 30 min at 37°C. The sections were then rinsed in 2 × SSC and submitted to the pre-hybridization–hybridization as described above.

Results

Expecting a low abundance of cystatin C mRNA in the heart, we used a sensitive RT-PCR method to establish that the cystatin C gene is expressed in cardiac tissues. Total RNA extracted from tissues was subjected to RT and the cDNAs were amplified with cystatin C-specific primers. The PCR resulted in amplification of a 339 bp fragment with RNAs extracted from the ventricle and atrium, indicating that the cystatin C gene is expressed in cardiac tissues. For comparison and as a positive control, cystatin C fragment was also amplified starting with RNAs from the liver and submandibular gland. RNA was also isolated from the same organs 48 hr after the injection of turpentine. In all instances, a 339 bp fragment, as predicted from the sequence of rat cystatin C cDNA (7), was amplified (Figure 1).

To further characterize cystatin C gene expression in the heart, Northern blot hybridizations were performed using a PCR-generated digoxigenin-labeled probe. Under stringent hybridization and washing conditions the probe hybridized to a single mRNA species of

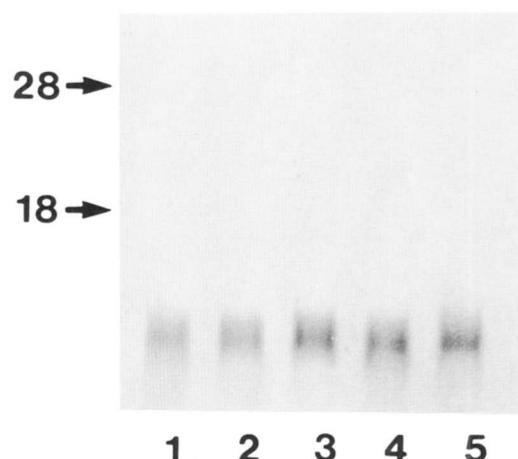


Figure 2. The effect of fasting and fasting + turpentine injection on the level of cystatin C mRNA in the heart. Northern blot hybridization to digoxigenin-labeled cystatin C probe was carried out using total RNA extracted from the hearts as described in Materials and Methods. The probe hybridized to a single mRNA species of about 700 bp. Neither fasting nor acute inflammation had an effect on the level of cystatin C mRNA in the heart. Arrows indicate the positions of 28S and 18S ribosomal RNAs on the blot. Lane 1, control; Lane 2, 24-hr fasting; Lane 3, 24-hr fasting + turpentine; Lane 4, 48-hr fasting; Lane 5, 48-hr fasting + turpentine.

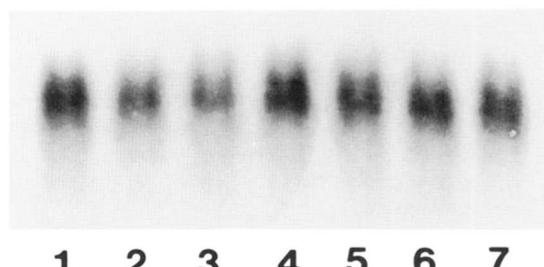


Figure 3. Effect of the β-adrenergic agonist IPR on the level of cystatin C mRNA in the heart. Northern blot hybridization to total RNA extracted from the hearts was carried out as described in Materials and Methods. IPR had no effect on the level of cystatin C mRNA. Lane 1, control; Lane 2, 1-hr IPR; Lane 3, 4-hr IPR; Lane 4, 18-hr IPR; Lane 5, 24-hr IPR; Lane 6, IPR for 6 days; Lane 7, IPR for 6 days followed by a recovery period of 6 days.

about 700 nucleotides. Fasting for 24 or 48 hr or acute inflammation produced by turpentine injection for 24 hr or 48 hr had no obvious effect on cystatin C mRNA levels in the heart (Figure 2). Nor was the level of the message altered in the hearts of rats that received a single injection of IPR 1, 4, 18, or 24 hr earlier or were treated with IPR for 6 days (Figure 3).

Next we performed *in situ* hybridizations using digoxigenin-labeled probes generated by PCR to identify the cellular site(s) of expression of the cystatin C gene in the heart. As a comparison, *in situ* hybridizations were also carried out on formalin-fixed cryostat sections of seminal vesicle known to contain one of the highest concentrations of cystatin C in the rat (14,18) and also in humans (2).

In the seminal vesicle a strong hybridization signal was seen in the cytoplasm of the epithelial cells. Very weak staining seen in smooth cells is probably not specific (Figure 4A). In the heart the signal was restricted to muscle fibers. Blood vessels, connective tis-

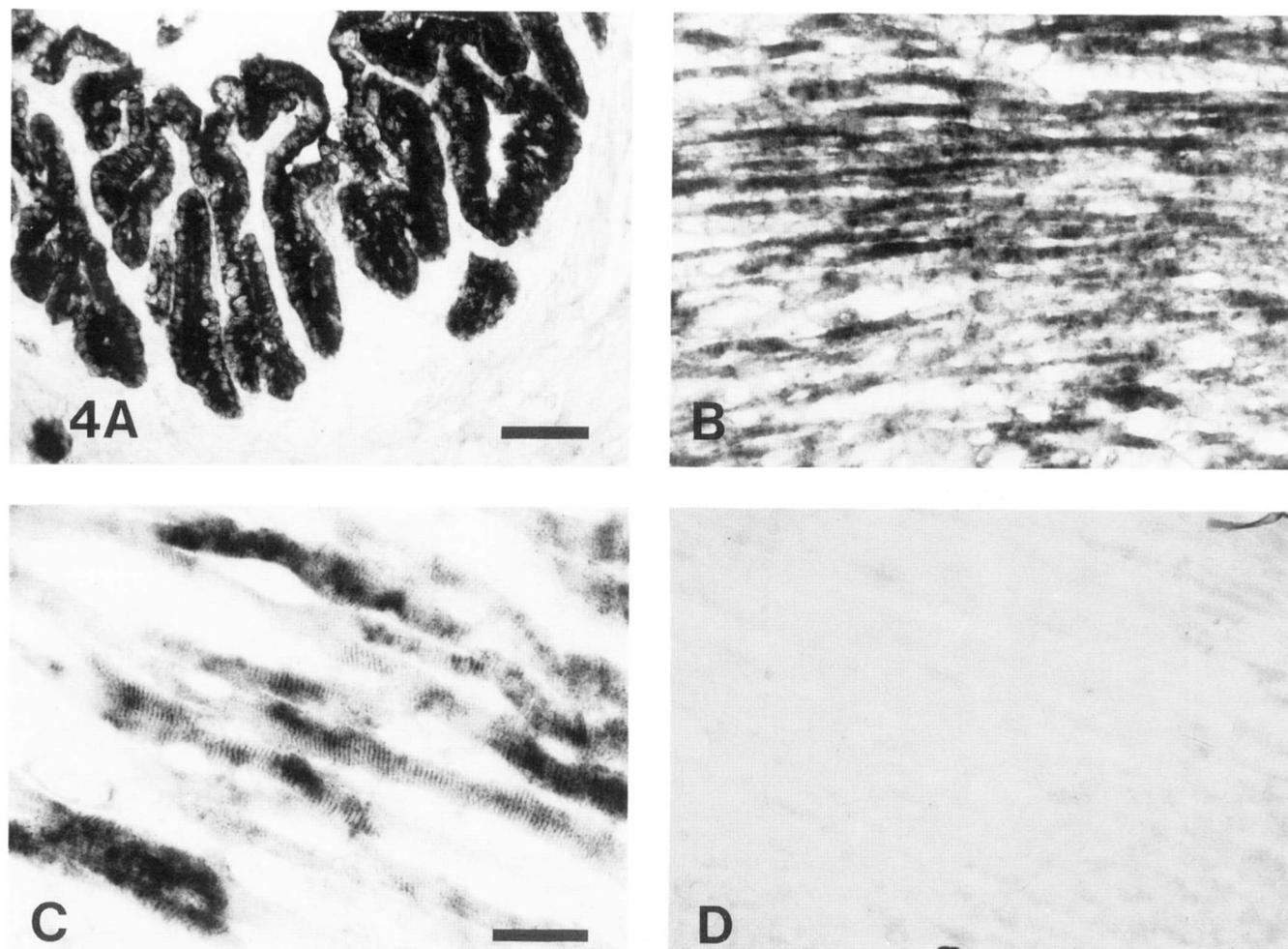


Figure 4. In situ hybridization of cystatin C mRNA. Formalin-fixed cryostat sections were hybridized with DIG-labeled cystatin C DNA probe as described in Materials and Methods. (A) Seminal vesicle used as a positive control. Strong signal is seen in the cytoplasm of epithelial cells. The very weak staining in the smooth muscle and connective tissue cells is of questionable specificity. The control preparation (not shown) was entirely negative. (B,C) Heart. The signal is localized to the cardiac muscle fibers. The intensity of staining varies from myocyte to myocyte. Separation of muscle fibers in C is an artifact of cryostat sectioning. (D) Heart, control preparation. The section was treated with RNase before pre-hybridization. No specific signal is seen. Bars: A,B,D = 100 μ m; C = 40 μ m.

sue cells, and endocardial and pericardial structures were negative. The intensity of staining, best observed in fibers cut longitudinally, varied. This variation was also seen in fibers cut across. Frequently, deposits of the reaction product, formazan, resulted in an accentuation of cross-striation (Figures 4B and 4C). Whether this pattern is artifactual or has significance is unclear. The control preparations, omitting the probe from the hybridization mixture or treating the section with RNase before hybridization, resulted in preparations essentially devoid of reaction product (Figure 4D).

Discussion

Cysteine proteinases that include a number of cathepsins, calpains, and the less well-characterized multicatalytic and metal-dependent proteinases (4) are involved in the initial stages of intra- and extracellular degradation and turnover of proteins and in the processing of prohormones. The activities of these enzymes are pre-

sumably controlled by inhibitory proteins, cystatins. It is now recognized that cystatins belong to a superfamily comprising the 1. stefins, 2. cystatins, and 3. kininogens (3,19). Cystatins (Family 2) occur intracellularly and extracellularly in various tissue fluids such as spinal fluid, tears, saliva, synovial fluid, milk, and seminal plasma (1).

Although the presence of thermostable inhibitors of cathepsins B and H in rat heart was first reported as early as 1979 by Lenney et al. (10), cardiac cystatins and the expression of cystatin genes in the heart have not been investigated. Using RT-PCR and Northern blot hybridizations we have now established that cystatin C is expressed in the heart of the rat. With RT-PCR, cystatin C mRNA was also detected in the submandibular gland and in the liver. Cole et al. (7) reported low levels of cystatin C mRNA in the submandibular gland but only traces in the liver as revealed by Northern blot hybridizations.

The regulation of the expression of the cystatin C gene(s) is not

known. Since acute inflammation stimulates the expression of T-kininogen genes (Family 3 of cystatins) in the liver (5,8) and β -adrenergic receptors are involved in the regulation of the cystatin S gene (Family 2 of cystatins) in the submandibular gland of the rat (16,17), we surmised that the cystatin C gene may be under similar regulatory mechanisms. However, neither acute inflammation nor administration of the β -adrenergic agonist isoproterenol had an effect on the level of cystatin C mRNA in the heart. In humans the cystatin C gene is also widely expressed, and Abrahamson et al. (2) suggested that this apparently non-tissue-specific expression is related to the structure of the gene's 5'-flanking region, which shares some properties with the promoter region of "housekeeping genes." Characterization of the rat cystatin C gene(s) is a prerequisite to further analyze the regulation of the expression of this gene.

In situ hybridizations using a digoxigenin-labeled probe revealed that cystatin C is primarily expressed in cardiac muscle fibers but not in connective tissue, endothelial cells, or smooth muscle cells. The specificity of the probe for cystatin C is indicated by the following: (a) the size of the probe was as predicted from the sequence of the rat cystatin C cDNA (7); (b) the probe did not hybridize to cystatin S mRNA, which has a 48% similarity to cystatin C mRNA (data not shown); (c) the probe hybridized to a single species of mRNA of about 700 nucleotides on Northern blots; (d) it revealed cystatin C mRNA in the seminal vesicle, known to contain high levels of cystatin C (a positive control); (e) the signal was abolished by RNase treatment of the sections; and (f) there was no staining if the probe was omitted from the hybridization mixture.

The role of cystatin C in the heart under physiological and pathological conditions remains to be established. The structure of the rat cystatin C cDNA (7) suggests that cystatin C is a secretory protein. Whether the inhibitor is released by the muscle fibers under physiological conditions or acts intracellularly is not known. It is conceivable, however, that under pathological conditions, particularly in ischemia, the inhibitor is released and plays a role in modulating the activities of the extracellular cathepsins derived from myocytes and/or inflammatory cells. In addition, under such conditions, cystatin C or its fragment may also affect the chemotactic and phagocytic functions of granulocytes (11,12).

Cystatin C may not be the only cysteine proteinase inhibitor in the heart. We have isolated T-kininogen from rat heart, which is not only a potent cysteine proteinase inhibitor but also a source of bradykinin (T-kinin). In contrast to cystatin C, however, the T-kininogen gene is not expressed in the heart, and the T-kininogen isolated from the heart is derived from the blood (Barka and van der Noen, submitted for publication). Furthermore, Katunuma and Kominami (9) found low levels of cystatin- α and much higher levels of cystatin- β (Family 1 of cystatins) in the heart. However, cystatin- β was localized chiefly in macrophages and not in cardiac muscle fibers. Although the level of cystatin inhibitory activity, measured by titration of papain inhibition, was increased in hearts enlarged by the infusion of isoproterenol (unpublished observations), the data presented here indicate that stimulation of the expression of kininogen or cystatin C genes does not seem to be involved in this process. Nevertheless, further studies of cardiac cystatins and their possible roles in cardiac hypertrophy, inflammation, and necrosis are warranted.

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