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Chondrocyte Cultures Express Matrix Metalloproteinase mRNA and Immunoreactive Protein; Stromelysin-1 and 72 kDa Gelatinase Are Localized in Extracellular Matrix Vesicles

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Abstract Previous studies have shown that costochondral cartilage cell cultures produce extracellular matrix vesicles which contain metalloproteinase activity. In the present study, we examined whether two matrix metalloproteinases (MMPs) known to be present in cartilage, stromelysin-1 and 72 kDa gelatinase, are expressed by fourth passage resting zone and growth zone costochondral chondrocytes and whether they are specifically incorporated into matrix vesicles produced by the cells. We also examined whether the cells synthesize tissue inhibitor of metalloproteinase-1 and -2 (TIMP-1 and TIMP-2). Oligonucleotide primers for stromelysin-1, 72 kDa gelatinase, tissue inhibitor of metalloproteinases-1 and -2 (TIMP-1 and TIMP-2), and GAPDH were synthesized and optimized for use in the reverse transcription-polymerase chain reaction (RT-PCR). It was found that both resting zone and growth zone chondrocytes produced mRNA for both MMPs and the two TIMPs. Further, immunostaining of cell layers with antibodies to 72 kDa gelatinase and stromelysin-1 showed that both cell types produced these MMPs in culture. Substrate gel electrophoresis and Western analysis were used to characterize MMP activity in matrix vesicles, media vesicles, or plasma membranes as well as in conditioned media produced by the chondrocyte cultures. It was found that matrix vesicles but not plasma membranes or media vesicles were selectively enriched in stromelysin-1. Also, 72 kDa gelatinase was found in matrix vesicles, but to a lesser extent than seen in media vesicles. The relative activity of each enzyme detected was cell maturation–dependent. No MMP activity was detected in conditioned media produced by either cell type. The results of this study show that MMPs are expressed by resting zone and growth zone chondrocytes in culture and differentially distributed among three different membrane compartments. This suggests that, in addition to the well-known activators and inhibitors of MMP activity in the matrix, differential membrane distribution may enable more precise control over the site, rate, and extent of matrix degradation by the cell.

Key words: metalloproteinases, growth plate cartilage, chondrocytes, matrix vesicles, RT-PCR, zymography, stromelysin-1, 72 kDa gelatinase

Chondrocytes derived from the resting zone and growth zone of costochondral cartilage have been used as a model for examining the role of cell maturation on the response of cartilage cells to a variety of regulatory factors [Boyan et al., 1992]. These studies have shown that the cells retain a differential phenotype in culture through four passages with respect to extracellular matrix synthesis [Schwartz et al., 1989], basal calcium ion flux [Langston et al., 1990; Schwartz et al., 1991], vitamin D metabolite production [Schwartz et al., 1992a], prostaglandin production [Schwartz et al., 1992b], phospholipid metabolism [Schwartz and Boyan, 1988; Schwartz et al., 1990; Swain et al., 1992], steroid hormone receptors [Nasatzky et al., 1994], and response to growth factors [Schwartz et al., 1992a, 1993], and hormones [Boyan et al., 1988a; Nasatzky et al., 1993; Schwartz et al., 1988a,b]. Particularly striking is the differential responsiveness of these two cell types to vitamin D metabolites. In general, growth zone chondrocytes respond to 1,25-

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$(OH)_2D_3$, whereas resting zone chondrocytes respond primarily to 24,25-$(OH)_2D_3$ [Boyan et al., 1988a; Nasatzky et al., 1993; Schwartz et al., 1988b, 1989, 1990; Sylvia et al., 1993]. In vivo studies support this cell maturation–dependent response to vitamin D metabolites [Atkin et al., 1985; Dean et al., 1985, 1989].

Both types of chondrocytes produce extracellular matrix vesicles in culture. The matrix vesicles and plasma membranes isolated from the cartilage cell cultures have been well characterized biochemically. Like matrix vesicles produced by cells in the resting zone and growth zone cartilage in vivo [Ali et al., 1970; Anderson, 1969; Bonucci, 1969; Thyberg and Friberg, 1970], matrix vesicles isolated from the cultures are biochemically distinct from the plasma membranes of their parent chondrocytes. Differences include phospholipid composition [Boyan et al., 1988b] and metabolism [Schwartz and Boyan, 1988; Schwartz et al., 1992b] and enzyme activities [Boyan et al., 1988a; Schwartz et al., 1988a,b]. Not surprisingly, the basal membrane fluidity of matrix vesicles is distinct from that of the plasma membrane [Swain et al., 1993], reflecting differences in structure and chemical composition. There are cell maturation–specific differences in matrix vesicle phospholipid composition [Boyan et al., 1988b] and alkaline phosphatase and phospholipase A_2 specific activities [Boyan et al., 1988a; Schwartz and Boyan, 1988] as well. Moreover, matrix vesicle enzyme activity is regulated differently than that of the plasma membrane when cells are exposed to a variety of regulatory factors, and these effects are also dependent on the state of chondrocyte maturation [Schwartz and Boyan, 1988; Schwartz et al., 1988a, 1992b, 1993; Yang et al., 1991].

Matrix vesicles are postulated to be involved in the maturation of the cartilage extracellular matrix. Hirschman et al. [1983] noted that neutral proteases are present in matrix vesicles isolated from fracture callus and suggested that they may participate in matrix modification. Katsura and Yamada [1986] have made similar observations in an avian model. Recently, we found that matrix vesicles contain high levels of active metalloproteinases capable of degrading proteoglycans [Dean et al., 1992]. The metalloproteinase activity in the plasma membrane fractions was significantly less than that found in matrix vesicles, and those matrix vesicles from growth zone chondrocyte cultures contained significantly more enzyme activity than those from resting zone chondrocyte cultures.

Taken together, these results suggest that matrix vesicles might be directly involved in preparing the matrix for bulk phase calcification by removing the inhibitory effects of proteoglycans in the matrix. This hypothesis is supported by the fact that matrix vesicles can reverse the inhibition of calcification in gelatin gels containing proteoglycan aggregates [Boskey et al., 1992] and that the level of metalloproteinases in matrix vesicles is regulated by exogenous factors which stimulate von Kossa–positive nodule formation in vitro [Dean et al., 1994]. The fact that resting zone chondrocytes, which do not calcify their matrix in vivo, also produce matrix vesicles containing metalloproteinases suggests that, in addition to preparing matrix for calcification, matrix vesicles may be involved in normal extracellular matrix remodeling as well.

The exact identity of the metalloproteinase(s) in matrix vesicles is unknown but is believed to belong to the matrix metalloproteinase (MMP) class of proteinases. The members of this group of proteinases are secreted into the extracellular matrix in latent form and require activation by enzymes such as plasmin for activity. In addition, they require both zinc and calcium ions for activity, have optimal activity at neutral pH, degrade extracellular matrix macromolecules, and are inhibited by tissue inhibitors of metalloproteinases (TIMP) [Woessner, 1991]. Interstitial collagenase (MMP-1), 72 kDa gelatinase (MMP-2), and stromelysin-1 (MMP-3) are members of this group and have been found in cartilage and implicated in normal matrix remodeling. They have also been implicated in the pathogenesis of several diseases affecting cartilage, such as osteoarthritis. At present, the localization and regulation of MMP activity in the matrix is largely unknown.

Matrix vesicle composition and activity is regulated both genomically during production and nongenomically via secretion of autocrine mediators which interact directly with the matrix vesicles in the extracellular matrix [Boyan et al., 1994]. A differential targeting of MMPs to matrix vesicles would provide more precise control over the site, rate, and extent of matrix degradation by the cell. The present study is based on the hypothesis that chondrocytes in culture produce stromelysin-1 and 72 kDa gelatinase, as well as TIMP-1 and TIMP-2, in a cell maturation–dependent manner and that stromelysin-1 and 72 kDa gelatinase are specifically localized to the

extracellular matrix vesicles, where they are stored in active form. To test this hypothesis, we first determined if mRNAs for 72 kDa gelatinase, stromelysin-1, TIMP-1, and TIMP-2 were present in cultures of resting zone and growth zone chondrocytes; then, by immunochemistry, we determined if the cell layers stained for these enzymes. With this information in hand, we isolated plasma membranes, matrix vesicles, and media vesicles (membrane vesicles released into the culture media during culture) [Boyan et al., 1988a,b] from both types of cultures and characterized the enzyme activity present in each fraction by substrate gel electrophoresis and Western blots.

MATERIALS AND METHODS

Reagents

All tissue culture reagents were obtained from GIBCO (Grand Island, NY). Guanidine thiocyanate and diethylpyrocarbonate were purchased from Fluka Chemical Corporation (Ronkonokoma, NY) and molecular biology–grade phenol from Bethesda Research Laboratories (Gaithersburg, MD). Avian myeloblastosis virus (AMV) reverse transcriptase was purchased from Life Sciences, Inc. (St. Petersburg, FL), and recombinant *Thermus aquaticus* DNA polymerase was obtained from Perkin Elmer Cetus (Norwalk, CT). RNasin ribonuclease inhibitor was obtained from Promega Corporation (Madison, WI), and 2′-deoxynucleoside 5′-triphosphate (dNTP) kits were purchased from Pharmacia LKB Biotechnology, Inc. (Piscataway, NJ). [γ-^{32}P]ATP (10.0 mCi/ml) was purchased from New England Nuclear (Boston, MA). T_4 polynucleotide kinase was purchased from Gibco, BRL (Gaithersburg, MD). Phenylmethylsulfonylfluoride (PMSF), 1,10-phenanthroline, and aminophenylmercuric acetate (APMA) were purchased from Sigma Chemical Company (St. Louis, MO), and 4-(2-aminoethyl)-benzenesulfonylfluoride (AEBSF) and α_2-antiplasmin were purchased from Calbiochem (San Diego, CA). Recombinant human stromelysin-1 was obtained from Dr. Scott Wilhelm of Bayer AG (West Haven, CT). Protein kinase C was obtained from Gibco BRL (Gaithersburg, MD). All other reagents were of analytical grade or the highest grade available.

Cell Culture

The culture system used in this study has been described in detail previously [Boyan et al., 1988b]. Briefly, rib cages were removed from 125 g male Sprague-Dawley rats by sharp dissection and placed in Dulbecco's Modified Eagle's Medium (DMEM) until microdissection could be performed. The resting zone and adjacent growth zone cartilage was separated, and care was taken to dissect out intervening tissue so that cross-contamination of cell zones would be decreased. When the dissection was complete, cartilage from each zone was sliced, placed in DMEM containing 10% fetal bovine serum (FBS), 1% antibiotic-antimycotic (penicillin-streptomycin-fungizone), and incubated overnight. The DMEM was then replaced by Hank's balanced salt solution (HBSS), followed by sequential incubations with 1% trypsin and 0.02% collagenase. After enzymatic digestion of the extracellular matrix was complete, cells were collected, resuspended in DMEM, and plated at an initial density of 10,000 cells/cm^2 for resting zone cells or 25,000 cells/cm^2 for growth zone cells. Cells were incubated in DMEM containing 10% FBS, 1% antibiotic-antimycotic, and 50 μg/ml sodium ascorbate in an atmosphere of 5% CO_2 and 100% humidity at 37°C for 24 h. The culture medium was replaced at that time and then at 72 h intervals until the cells reached confluence. At confluence, cells were subcultured to T75 flasks at the same plating densities as before and allowed to return to confluence. Fourth passage cells were used for all experiments. Previous studies have shown that these cells retain their chondrocytic phenotype and differential responsiveness to growth factors and hormones through this passage in culture [Boyan et al., 1988a,b, 1990, 1992; Schwartz and Boyan, 1988].

Cervical carcinoma (HeLa) and rhabdomyosarcoma (RD) cells were obtained from the American Type Culture Collection (Rockville, MD) and cultured as previously described [Chen et al., 1992]. FDC-P cells, a murine hemopoietic cell line, were obtained from Dr. Sheri Abboud (Department of Medicine, University of Texas Health Science Center at San Antonio) and cultured as previously described [Dexter et al., 1980].

Isolation of Media Vesicles, Matrix Vesicles, and Plasma Membranes

At harvest, conditioned media were centrifuged at 500*g* for 5 min to pellet unattached cells. The supernatant was recentrifuged at 21,000*g* for 10 min to pellet cellular debris, including mitochondria or endoplasmic reticulum. The resulting supernatant was centrifuged

at 100,000*g* for 1 h to pellet media vesicles which were resuspended in either 0.9% NaCl, 0.9% NaCl containing 0.5 mM PMSF, or phosphate-buffered saline, pH 7.4, containing 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, and 0.5 mM PMSF (PBS-TDS) and stored at −70°C.

The cell layer, including extracellular matrix and cells, was washed with HBSS and then trypsinized (1% trypsin in HBSS). After 10 min, the trypsin was inactivated by addition of an equal amount of DMEM containing 10% FBS and antibiotic-antimycotic, with or without 0.5 mM PMSF. Cells were separated from the trypsin digest by centrifugation at 500*g* for 10 min and plasma membranes prepared from the resulting cell pellet as previously described [Fitzpatrick et al., 1969] and stored in 0.9% NaCl at −20°C. The supernatant of the 500*g* centrifugation of the trypsin digest was centrifuged again at 21,000*g* for 10 min to pellet cell debris. The resulting supernatant was centrifuged at 100,000*g* for 1 h to pellet matrix vesicles that were washed with 0.9% NaCl and recentrifuged at 100,000*g* [Boyan et al., 1988b]. The matrix vesicle pellet was resuspended as described for media vesicles.

All membrane fractions were assayed for protein content using the bicinchoninic acid (BCA) reagent (Pierce Chemical Co., Rockford, IL) and for alkaline phosphatase using para-nitrophenylphosphate as a substrate at pH 10.2 [Bretaudiere and Spillman, 1984] to ensure that all three types of membranes behaved as previously described [Boyan et al., 1988a,b, 1990; Dean et al., 1992; Schwartz and Boyan, 1988]. The purity of the membrane fractions using this methodology is well documented [Boyan et al., 1988a,b; Dean et al., 1992; Schwartz and Boyan, 1988; Schwartz et al., 1988a,b; Swain et al., 1993].

Extraction of Matrix Vesicle Enzymes

Extraction of matrix vesicle enzymes was performed as previously described [Dean et al., 1992], with slight modification. Matrix vesicles (700 μl) were mixed with an equal volume of extraction buffer (0.1 M Tris, pH 7.4, containing 4 M guanidine HCl, 0.02 M $CaCl_2$, and 0.4% Triton X-100) with or without 0.5 mM PMSF, sonicated at 4°C for 10 min, and then placed on a tilt plate for 2 h at 4°C. Samples were centrifuged at 105,000*g* for 1 h at 4°C and the supernatants dialyzed overnight into PBS (pH 7.4).

Matrix Metalloproteinase Gene Expression

Synthesis of oligonucleotide primers. Rat cDNA sequences were used for designing oligonucleotide primers for stromelysin-1 and 72 kDa gelatinase [Breathnach et al., 1987; Marti et al., 1993]. Rat primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [Forte et al., 1985] and aldolase B [Tsutsumi et al., 1984] were also constructed. Since the cDNAs for rat tissue inhibitor of metalloproteinases-1 (TIMP-1) and TIMP-2 had not been reported, we used the human cDNA sequences for TIMP-1 [Docherty et al., 1985] and TIMP-2 [Carmichael et al., 1986]. The PCR products, resulting from the use of these primers with our rat RNA, were electrophoresed on agarose gels, extracted, sequenced, and found to be homologous with the reported sequences for both human and mouse TIMP-1 (96%) and TIMP-2 (92%). This indicated that the TIMP-1 and TIMP-2 primers targeted the desired rat gene products.

Primer design was accomplished with Oligo computer software (version 4.0; National Biosciences, Plymouth, MN). All primers were designed to be 25 nucleotides long and approximately 50% G + C rich; in addition, they were to have a low ΔG at their 3′ end and a T_m within 5°C of their optimum annealing temperature and to exhibit no 3′-complementarity between primer pairs [Innis and Gelfand, 1990; Rappolee et al., 1989]. Oligonucleotide primers were synthesized by, and purchased from, Genosys Biotechnologies, Inc. (The Woodlands, TX). Their sequences and characteristics are shown in Tables I and II.

TABLE I. Sequences of 5′- and 3′-Oligonucleotide Primers

Gene	5′-primer/(5′ → 3′)	3′-primer/(5′ → 3′)
Stromelysin-1	ACT GGA TTT ACC AAG AGA GAG TGT G	CAT CGT TCA TCA TCA TCA AAG TGA G
GAPDH	TGA TGG GTG TGA ACC ACG AGA AAT A	AAC GGA TAC ATT GGG GGT AGG AAC A
TIMP-1	ATC CTG TTG TTG CTG TGG CTG ATA G	TGC TGG GTG GTA ACT CTT TAT TTC A
TIMP-2	AAA CGA CAT TTA TGG CAA CCC TAT C	ACA GGA GCC GTC ACT TCT CTT GAT G
72 kDa gelatinase	CCT GAT GCT GAT ACT GAC ACT GGT A	CCA TTT TCT TCT TCA CTT CAT TGT A

TABLE II. Characteristics of Oligonucleotide Primers*

Gene	% G:C content	Optimal annealing temperature (°C)	PCR annealing temperature (°C)	Cycles	Mix
Stromelysin-1	49	54.8	56	28	A_1/T_2
GAPDH	56.3	59.3	64	22	A_2/T_2
TIMP-1	56.6	59.2	64	35	A_1/T_1
TIMP-2	54.7	58.4	64	20	A_1/T_1
72 kDa gelatinase	52.3	56.3	56	20	A_1/T_1

*%G:C is percent guanine:cytosine nucleotides in the primers. For composition of A and T mixes see Materials and Methods.

Reverse transcription–polymerase chain reaction (RT-PCR). Reverse transcription was performed by adding 1 μg total RNA [Chomczysnki and Sacchi 1987] to 100 ng of 3′ primer, adjusting the total volume to 13.75 μl with diethylpyrocarbonate-(DEPC) treated water and heating to 68°C for 15 min. Samples were then chilled on ice and the final reaction volume adjusted to 20 μL by adding 6.25 μl of A_1 mix containing 20 units RNasin, 40 mM dithiothreitol, 128 mM KCl, 160 mM Tris-HCl (pH 8.3), 0.0256% gelatin, 25.64 mM $MgCl_2$, 1.6 mM of each dNTP, 5 μg bovine serum albumin, and 1.8 units AMV reverse transcriptase. The incubation was then continued for 1 h at 42°C. Some primer sets required optimization using an A_2 mix that contained all the reagents of the A_1 mix except that $MgCl_2$ was at 6.02 mM.

When reverse transcription was complete, samples were immediately used in the polymerase chain reaction. To the reverse transcription mixture, 78 μL of T_1 mix (= 10.3 mM Tris-HCl buffer, pH 9.0, containing 82 mM KCl, 3.07 mM $MgCl_2$, 0.26 mM of each dNTP, 0.01% gelatin, 0.1% Triton X-100, and 2.5 units Taq polymerase) and 2 μL (100 ng) of 5′-primer specific for either an MMP, TIMP, or GAPDH added. Some primer sets required optimization using T_2 mix which contained all the reagents of the T_1 mix except that 2.56 mM $MgCl_2$ and 7.6% DMSO were added. Also, 5′-oligonucleotide primers were end-labeled with [γ^{32}P]-ATP using T_4 kinase. The final reaction mixture was placed in a model 480 DNA Thermal Cycler (MJ Research, Watertown, MA) to conduct the polymerase chain reaction. The amplication profile involved denaturation at 94°C for 1.5 min, annealing at the primer-specific annealing temperature (Table II) for 1 min, extension for 1 min at 72°C, and denaturation at 95°C for 20 s. Sixteen microliters of the reaction mixture was removed at various cycles (16–38) of the PCR at the end of the annealing step and electrophoresed on 5% polyacrylamide gels in Tris/borate/EDTA buffer and stained with ethidium bromide. Appropriate bands were cut from the gel, placed in 5 ml of liquid scintillation fluid, and counted. The amount of radioactivity recovered from the bands was plotted against the number of PCR cycles to assess efficiency of the PCR and radioactivity from the MMP and GAPDH bands.

Matrix metalloproteinase gene expression. Total RNA was extracted from fourth passage cultures of resting zone and growth zone chondrocytes as described by Chomczynski and Sacchi [1987] and subjected to RT-PCR [Conboy et al., 1988; Rappolee et al., 1989] using the primers shown in Table I with the characteristics shown in Table II. PCR products were extracted from 1% agarose gels using a QIAEX gel extraction kit (Qiagen, Inc., Chatsworth, CA) and sequenced using a dsDNA cycle sequencing system (GIBCO BRL, Gaithersburg, MD) based on the Sanger method [Sanger et al., 1977]. Each primer pair gave amplicons of the expected base pair size, and sequences were verified through the National Center for Biotechnological Information (Bethesda, MD). Significance between treatment and controls was determined using Wilcoxon's signed rank test using $P < 0.05$.

Localization of Matrix Metalloproteinases by Immunohistochemistry

Immunohistochemistry was used to localize stromelysin-1 and 72 kDa gelatinase in resting zone and growth zone chondrocyte cell layers as previously described [Nasatzky et al., 1994]. Various cell lines or tissues (shown in the figures) were selected as controls based on prior reports in the literature or RT-PCR data indicating the presence or absence of expression for a particular MMP. Stromelysin-1 and 72 kDa gelatinase were purified according to the method of Bailey et al. [1994] and used as antigens for the preparation of sheep polyclonal antisera by

The Binding Site (San Diego, CA). Each antibody was obtained as an affinity-purified immunoglobulin and shown by double immunodiffusion to cross-react with rat stromelysin-1 or 72 kDa gelatinase.

Cell layers were washed with ice-cold PBS, fixed in neutral buffered formalin, and then removed from the flask by scraping. The cell layer pellet was isolated by centrifugation and then embedded in paraffin. Sections (5 μm) were cut, deparaffinized, and then incubated overnight with a 1:50 dilution of the primary antibody or relevant preimmune IgG in controls and visualized using sheep or mouse IgG Vectastain Elite ABC kits (Vector Laboratories, Burlingame, CA). Resting zone and growth zone chondrocytes were counterstained with hematoxylin; HeLa, FDC-P, and breast carcinoma cells were counterstained with methyl green. Specimens were photographed using an Olympus Vanox II photomicroscope.

Assay of Matrix Metalloproteinase Activity by Zymography

MMP activity in the matrix vesicle, plasma membrane, and media vesicle fractions, as well as in the 100,000*g* supernatants of conditioned media, was determined by substrate gel electrophoresis using casein or gelatin [Adler et al., 1990; Herron et al., 1986; Unemori and Werb, 1986]. With this method, zones of clearing indicate the presence of proteolytic enzymes. Further, the molecular weight of the proteinases can be estimated by comparison with molecular weight standards. Membrane fractions or their extracts (20–40 mg protein) were suspended in Laemmli sample buffer (0.06 M Tris-HCl, pH 6.8, buffer containing 0.025% bromphenol blue, 1% sucrose, and 2.5% SDS), loaded onto SDS-polyacrylamide gels (4.3% stacking gel; 12.5% resolving gel) containing 1 mg/ml of either casein or gelatin, and electrophoresed [Laemmli, 1970]. To resolve the M_r of only low molecular weight gelatinolytic activity, matrix vesicle proteins were electrophoresed as above and mobility compared to low molecular weight standards. After electrophoresis, gels were washed twice with 2.5% Triton X-100 and then incubated in 50 mM Tris-HCl, pH 7.6, buffer containing 5 mM $CaCl_2$ for 36 h at 37°C. Gels were stained with 0.5% Coomassie blue R250 and destained with 45% methanol/10% acetic acid.

To determine which class of proteinase was responsible for substrate digestion, some gels were incubated in buffer containing the metalloproteinase inhibitor, 1,10-phenanthroline (4 mM); the serine proteinase inhibitors, 4-(2-aminoethyl)-benzenesulfonylfluoride (AEBSF) (100 μM), PMSF (5 mM), or α_2-antiplasmin (10 ng/ml). In some instances, guanidine extracts were pretreated with 1 mM aminophenylmercuric acetate (APMA) to determine if further activation of the MMPs could be achieved [Adler et al., 1990]. Zinc (1 μM) was also added to the substrate digestion buffer in some gels to determine if further activation of the enzymes was possible.

Western Blotting of Matrix Metalloproteinases

Matrix vesicle, media vesicle, and plasma membrane fractions (containing 20–40 μg protein) were solubilized in Laemmli sample buffer and loaded onto SDS-polyacrylamide gels either directly or after immunoprecipitation with MMP antibody. In the latter instance, membrane protein was mixed with an equal volume of anti-MMP antibody (1:100 dilution) and incubated for 1 h on ice. To this mixture, an equal amount of Protein G-PLUS agarose (Oncogene Sciences, Inc., Uniondale, NY) was added and the incubation continued for an additional 18 h at 4°C. The samples were then washed three times with 200 μl cold PBS, and 25 μl Laemmli sample buffer was added to release the bound antigen. After the last centrifugation, the supernatant was decanted and boiled for 5 min, loaded onto the gel, and electrophoresed as described above.

Proteins on SDS gels were transferred to Polyscreen™ (polyvinylidine difluoride) transfer membranes (DuPont, Boston, MA) according to the method of Towbin et al. [1979]. Membranes were washed overnight with PBS-Tween 20, nonspecific binding sites blocked with 5% nonfat dry milk, and incubated with primary antibody (1:1000) for 1 h. After washing, the membrane was incubated with anti-sheep or anti-mouse IgG (1:1,000) in 1% PBS-Tween 20 for 1 h. Blots were developed with the Western Blot Chemiluminescence Reagent (DuPont, Boston, MA) and visualized on Reflection™ (NEF-496) autoradiography film (DuPont).

Verification of Stromelysin-1 in Matrix Vesicles

Recombinant human stromelysin-1 (100 ng) was incubated with 1 or 2 units of protein kinase

C in the presence of 1 μCi [^{32}P]-ATP [Sylvia et al., 1993]. Western blots of the recombinant stromelysin-1/protein kinase C mixtures were probed with anti-stromelysin-1 antibody and immunoreactive bands visualized as described above. In addition, phosphorylated protein was detected by conventional autoradiography of the transfer membrane using Kodak X-Omat film.

RESULTS

Matrix Metalloproteinase Gene Expression

By use of RT-PCR, the genes for stromelysin-1 (243 bp), 72 kDa gelatinase (457 bp), TIMP-1 (692 bp), and TIMP-2 (430 bp) were found to be constitutively expressed in resting zone and growth zone chondrocytes (Table III). The housekeeping gene, GAPDH, was found in both cell types, while aldolase B, another frequently used housekeeping gene, was undetectable. In all instances, RT-PCR revealed the presence of a single mRNA species for each of the MMP and TIMP genes in resting zone and growth zone chondrocytes (Fig. 1). The identified gene products, after extraction from the gel, exhibited >90% identity with their reported cDNA sequences.

Both cell types amplified the GAPDH gene to a similar extent during the exponential phase of amplification, demonstrating that GAPDH gene expression in resting zone and growth zone chondrocytes is similar (Fig. 2). There was differential expression of MMP mRNA in the resting zone and growth zone cells, however. Both cell types displayed similar levels of stromelysin-1 gene expression at 24 cycles, but by 26–32 cycles differences in resting zone and growth zone gene expression became apparent, suggesting that resting zone chondrocytes may contain more mRNA for this MMP than do growth zone chon-

TABLE III. MMP Gene Expression in Resting Zone and Growth Zone Chondrocytes*

Gene	RC	GC	PCR product (bp)	cDNA species
Stromelysin-1	X	X	243	rat
GAPDH	X	X	332	rat
TIMP-1	X	X	692	human
TIMP-2	X	X	430	human
72 kDa gelatinase	X	X	457	rat
Aldolase B	ND	ND	ND	rat

*GC = growth zone chondrocytes. ND = not detected. RC = resting zone chondrocytes.

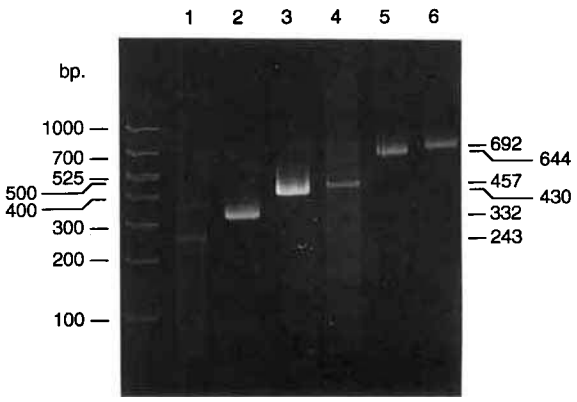


Fig. 1. Phenotyping of resting zone chondrocytes using RT-PCR. RT-PCR was performed on resting zone chondrocyte total RNA. PCR amplification was performed using primers (see Table I) for stromelysin-1, GAPDH, TIMP-1, TIMP-2, 72 kDa gelatinase, and interstitial collagenase (included on gel as part of another study). Samples were electrophoresed on 5% polyacrylamide gels to confirm amplicon size. Similar band sizes were observed for growth zone chondrocytes. *Lane 1:* Stromelysin-1. *Lane 2:* GAPDH. *Lane 3:* TIMP-2. *Lane 4:* 72 kDa gelatinase. *Lane 5:* Interstitial collagenase. *Lane 6:* TIMP-1. DNA base pair size standards are shown to the left. Base pair sizes of all amplicons are shown to the right.

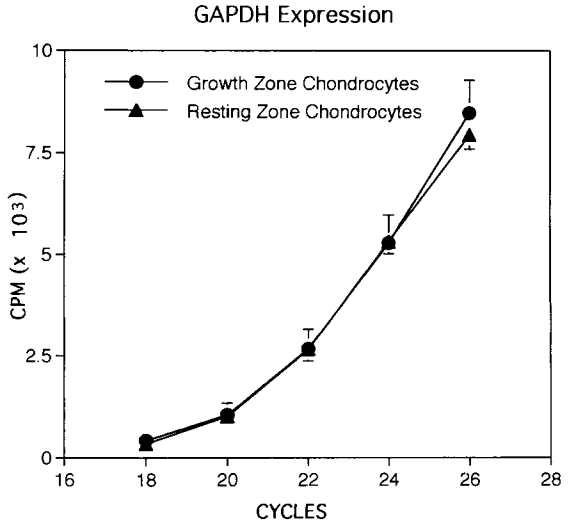


Fig. 2. GAPDH expression in resting zone and growth zone chondrocytes. Total RNA was extracted from resting zone and growth zone chondrocyte cell layers at 30 min after achieving confluence and RT-PCR performed using GAPDH primers. Equal aliquots were removed during the amplification phase to assess upregulation of GAPDH mRNA. Both cell types amplified the GAPDH gene during the exponential phase of amplification. This confirmed that GAPDH gene expression in resting zone and growth zone chondrocytes is similar. Data represent the mean ± SEM for an individual experiment (n = 3, T-75 flasks).

drocytes (Fig. 3). When data were adjusted for GAPDH, our housekeeping gene (Fig. 4), a two-fold higher content of stromelysin-1 mRNA was found in the resting zone cells. Both cell types also displayed similar levels of 72 kDa gelatinase gene expression at 14 and 16 cycles, but the level of resting zone chondrocyte mRNA for this enzyme was increased during the exponential phase of amplification (Fig. 5), suggesting that the resting zone cells may also express higher levels of this MMP. Adjustment for GAPDH levels showed a significant difference between the mRNA levels in the two cell types for this MMP, with that of the resting zone being higher (Fig. 6).

Immunolocalization of Matrix Metalloproteinases

MMPs were immunolocalized in cell layers of resting zone and growth zone chondrocytes by use of commercially available antibodies with known cross-reactivity to rat MMPs. Staining of both resting zone and growth zone chondrocyte cell layers by anti-stromelysin-1 antibody was predominantly observed in the extracellular matrix (Fig. 7A,B). The specificity of the staining was shown by three criteria. First, no staining was observed in resting zone chondrocyte cell layers when preimmune sheep IgG was substituted for the primary antibody in the staining procedure (Fig. 7C), indicating that any staining in the antibody-treated sections was not due to nonspecific adherence of immunoglobulin to the sections. Second, breast cancer biopsy tissue, previously shown to contain stromelysin-1, stained positively after incubation with the primary antibody (Fig. 7D) and negatively when preimmune sheep IgG was used (Fig. 7E). Third, RT-PCR failed to detect the gene for stromelysin-1 in HeLa cells (data not shown), and staining of these cells with anti-stromelysin-1 antibody was similarly negative (Fig. 7F), providing confirmation that, in the absence of enzyme, false positives were not detected.

Immunostaining was also positive for the presence of 72 kDa gelatinase in resting zone and growth zone chondrocyte cell layers (Fig. 8A,B). The specificity of the reaction was demonstrated by absence of staining when preimmune sheep

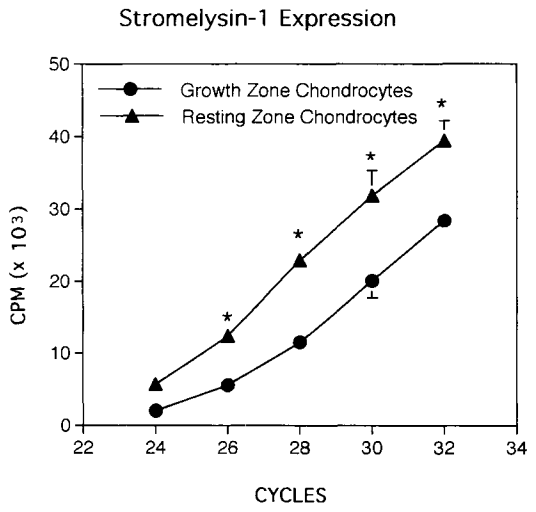


Fig. 3. Stromelysin-1 expression in resting zone and growth zone chondrocytes. At confluence, media were changed and resting zone and growth zone chondrocyte cell layers harvested 30 min later. Total RNA was extracted from these cell layers and RT-PCR performed using stromelysin-1 primers. Equal aliquots were removed during the amplification phase to assess upregulation of stromelysin-1 mRNA. Both cell types displayed similar levels of gene expression at 24 cycles, but the level of resting zone chondrocyte gene expression was increased during the exponential phase of amplification (cycle 26–32), suggesting that resting zone chondrocytes contain more mRNA for stromelysin-1 than growth zone chondrocytes. Data represent the mean ± SEM for an individual experiment (n = 3, T-75 flasks); $^*P < 0.05$, resting zone vs. growth zone.

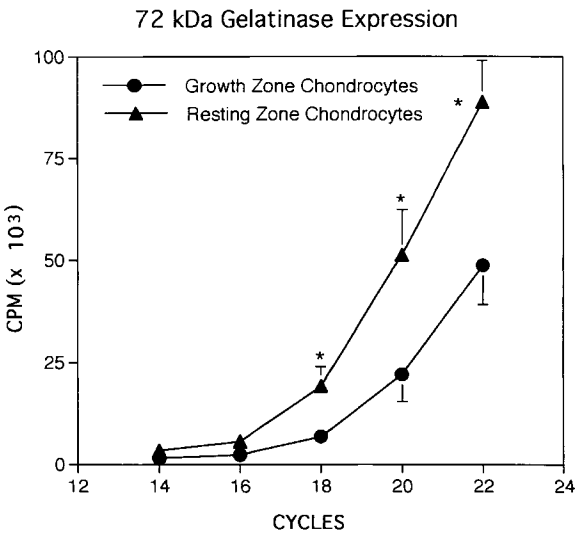


Fig. 4. Expression of 72 kDa gelatinase in resting zone and growth zone chondrocytes. Cells were treated and RNA isolated as described in Fig. 3. RT-PCR was then performed using 72 kDa gelatinase primers. Equal aliquots were removed during the amplification phase to assess upregulation of mRNA. Both cell types displayed similar levels of gene expression at 14 and 16 cycles, but the level of resting zone chondrocyte gene expression was increased during the exponential phase of amplification, suggesting that resting zone chondrocytes contain more mRNA for 72 kDa gelatinase than growth zone chondrocytes. Data represent the mean ± SEM for an individual experiment (n = 3, T-75 flasks); $^*P < 0.05$, resting zone vs. growth zone.

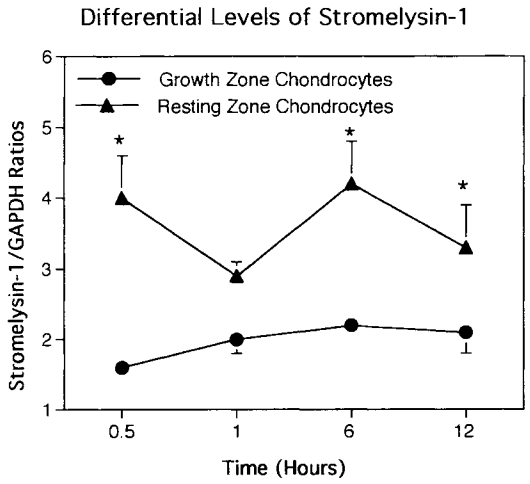


Fig. 5. Differential levels of stromelysin-1 in resting zone and growth zone chondrocytes. At confluence, media were changed and resting zone and growth zone cell layers harvested 30 min, 1 h, 6 h, or 12 h later. Total RNA was extracted and RT-PCR performed using stromelysin-1 and GAPDH primers. PCR products were electrophoresed in 5% polyacrylamide gels; appropriate bands were cut from the gel and counted. Data were analyzed as previously described [Chen et al., 1992] via equation 1. Resting zone chondrocytes displayed consistently elevated levels of mRNA for stromelysin-1 compared to growth zone chondrocytes. Data represent mean ± SEM for an individual experiment (n = 6, T-75 flasks); $^*P < 0.05$, resting zone vs. growth zone.

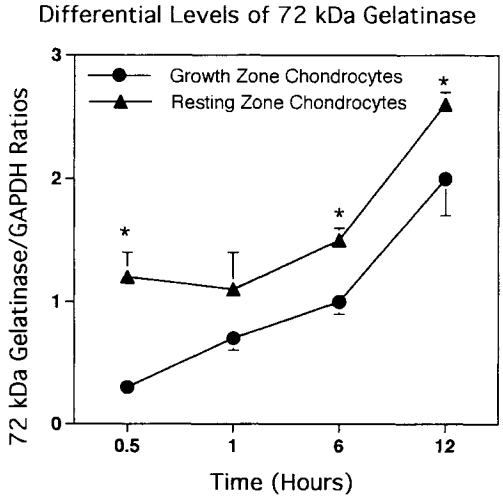


Fig. 6. Differential levels of 72 kDa gelatinase in resting zone and growth zone chondrocytes. At confluence, media were changed and resting zone and growth zone cell layers harvested 30 min, 1 h, 6 h, and 12 h later. Total RNA was extracted and RT-PCR performed using 72 kDa gelatinase and GAPDH primers. Data were calculated as described in Fig. 4. Resting zone chondrocytes displayed consistently elevated levels of mRNA for 72 kDa gelatinase compared to growth zone chondrocytes. Data represent the mean ± SEM for an individual experiment (n = 6, T-75 flasks); $^*P < 0.05$, resting zone vs. growth zone.

IgG was used in the staining protocol (Fig. 8C). Breast cancer tissue stained strongly (Fig. 8D) in both intracellular and extracellular locations; no stain was observed in the absence of primary antibody (Fig. 8E). FDC-P cells were negative for the presence of 72 kDa gelatinase by both immunohistochemistry (Fig. 8F) and RT-PCR (data not shown).

Identification and Localization of Matrix Metalloproteinases in Chondrocyte Membrane Fractions

Zymography revealed that matrix vesicles from both resting zone and growth zone chondrocytes contained enzymes of M_r 28–29 kDa that were active in digesting casein (Fig. 9A). Very low levels of caseinolytic activity were found in the plasma membranes, and none was detected in the media vesicles (data not shown). The appearance of one or two bands of activity on the gels was variable and depended on how the membranes were prepared and assayed after electrophoresis. Matrix vesicles that were isolated, mixed with sample buffer, electrophoresed, and then assayed without further treatment showed only one band of activity (Fig. 9A, lanes 2, 3). In contrast, matrix vesicles that were stored in saline (data not shown), incubated with APMA prior to electrophoresis and during the substrate digestion step (Fig. 9A, lanes 4, 5), or matrix vesicle guanidine extracts which were assayed on the gel without further treatment (Fig. 9A, lanes 6, 7), showed two major bands of activity. Those matrix vesicle guanidine extracts which were treated with APMA and then incubated with APMA during the substrate digestion step (Fig. 9A, lanes 8, 9) contained one or no bands of activity. This suggested that APMA did not further activate the enzymes. Matrix vesicles from growth zone chondrocyte cultures appeared to contain more enzyme activity than those from resting zone cell cultures, and matrix vesicles from both types of cultures contained enzyme that was largely in active form.

The addition of proteinase inhibitors to the buffer during substrate digestion was used to determine the class of proteinase responsible for casein digestion (Fig. 9B). It was found that AEBSF and α_2-antiplasmin, both serine proteinase inhibitors, had no effect on matrix vesicle enzyme activity in either the 48 kDa or the 28–29 kDa range (Fig. 9B, lanes 2–5). In contrast, 1,10-phenanthroline, a metalloproteinase inhibitor, blocked all matrix vesicle enzyme ac-

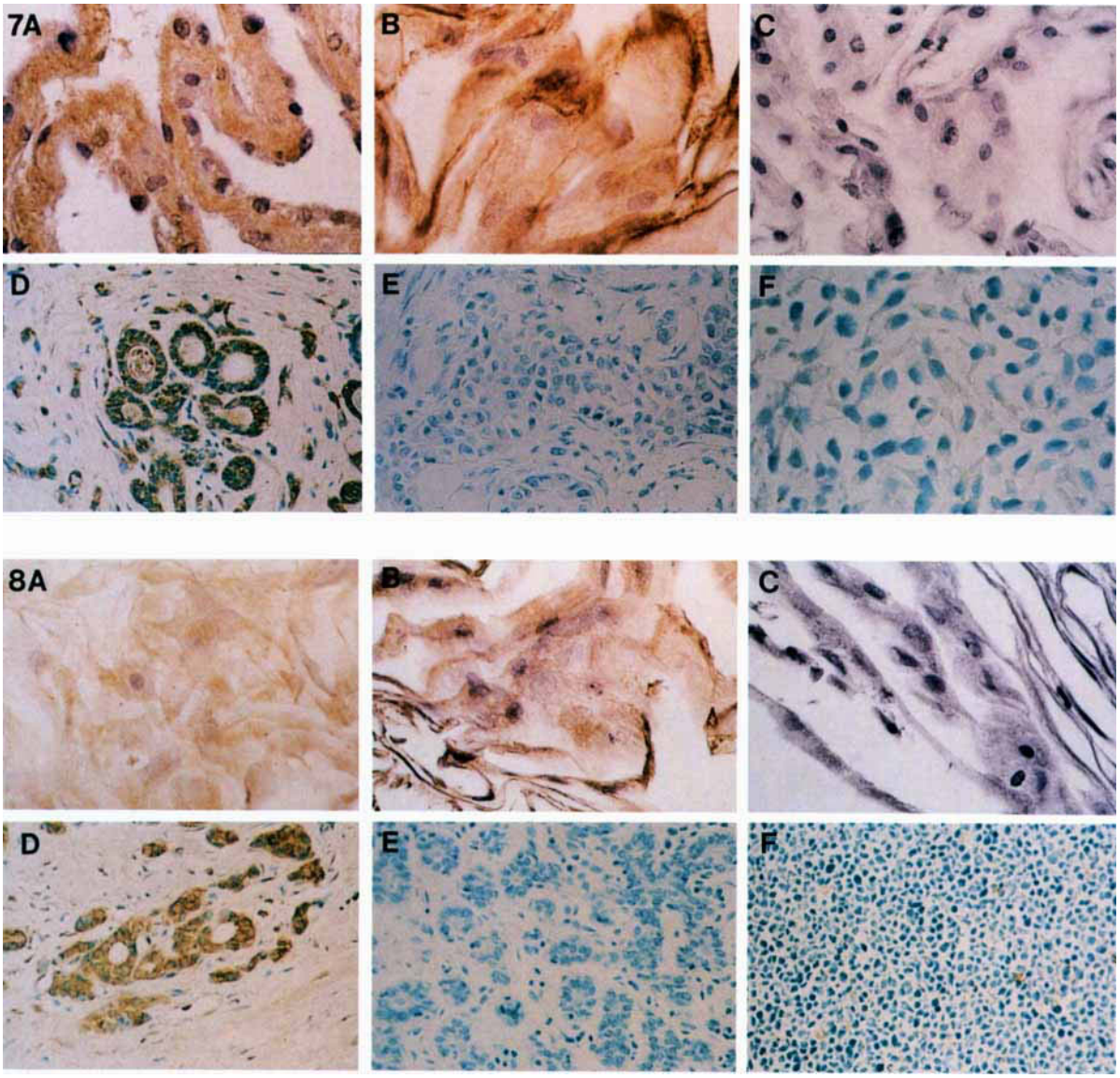


Fig. 7. Immunohistochemical localization of stromelysin-1 in resting zone and growth zone chondrocyte cultures. **A:** Resting zone chondrocytes stained with anti-stromelysin. ×165. **B:** Growth zone chondrocytes stained with anti-stromelysin. ×165. **C:** Resting zone chondrocytes incubated with preimmune IgG. ×165. **D:** Breast cancer tissue stained with anti-stromelysin. ×100. **E:** Breast cancer tissue incubated with preimmune IgG. ×100. **F:** HeLa cells stained with anti-stromelysin. ×165.

Fig. 8. Immunohistochemical localization of 72 kDa gelatinase in resting zone and growth zone chondrocyte cultures. **A:** Resting zone chondrocytes stained with anti-72 kDa gelatinase. ×165. **B:** Growth zone chondrocytes stained with anti-72 kDa gelatinase. ×165. **C:** Growth zone chondrocytes stained with preimmune IgG. ×165. **D:** Breast cancer tissue stained with anti-72 kDa gelatinase. ×100. **E:** Breast cancer tissue incubated with preimmune IgG. ×100. **F:** FDC-P cells stained with anti-72 kDa gelatinase. ×100.

tivity in the 48 kDa and 28 kDa regions of the gel and partially blocked the activity found in the 29 kDa region (Fig. 9B, lanes 6, 7). When matrix vesicles and their extracts were immunoprecipitated with anti-stromelysin antibody prior to zymography of the immunoprecipitated protein, only the 29 kDa region of the zymogram showed activity (data not shown). Inclusion of serine proteinase inhibitors in the storage and extraction buffers, as well as overloading the lanes, was necessary for detection of metalloproteinase activity in the M_r 48 kDa region of the gel (Fig. 9B, lanes 2–5), indicating that serine proteinases may have been present in the matrix vesicle extracts.

Substrate specificity, molecular weight, and inhibition by 1,10-phenanthroline suggested that the activity in matrix vesicles was stromely-

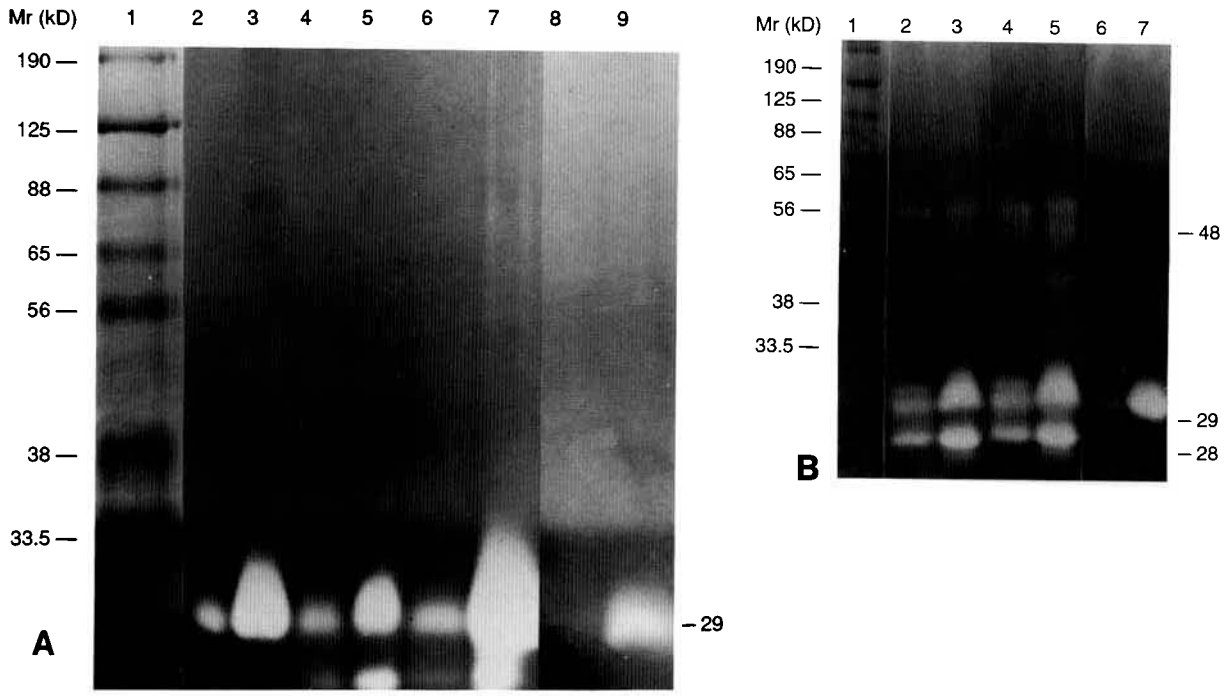
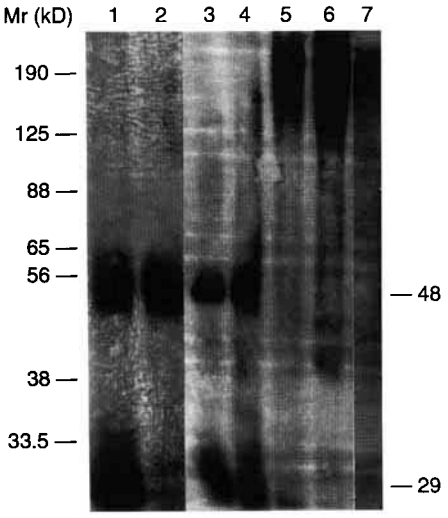
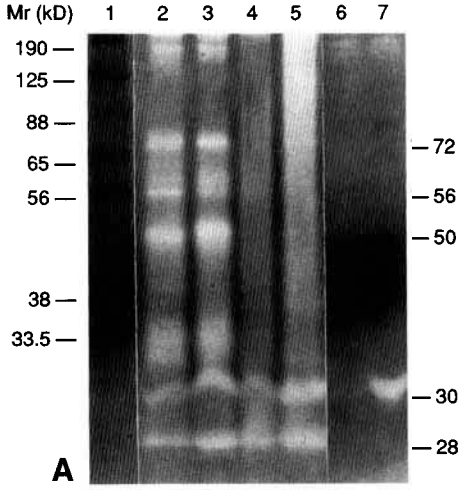


Fig. 9. Casein zymography of resting zone and growth zone chondrocyte matrix vesicles. Resting zone and growth zone chondrocyte matrix vesicle proteins were separated on 12.5% polyacrylamide gels containing SDS and 1 mg/ml casein. The presence of MMPs was determined by incubating the gels in 50 mM Tris buffer, pH 7.6, containing 5 mM $CaCl_2$ for 36 h at 37°C after removal of the SDS. After the incubation, the gel was stained with Coomassie blue. Clear bands indicate the presence of proteolytic enzymes. **A:** *Lane 1:* Molecular weight markers. *Lanes 2, 3:* Resting zone (lane 2) and growth zone (lane 3) chondrocyte matrix vesicles (20 μg protein) electrophoresed immediately after isolation without any additional treatment. *Lanes 4, 5:* Resting zone (lane 4) and growth zone (lane 5) chondrocyte matrix vesicles (20 μg protein) incubated with 1 mM APMA prior to electrophoresis and during the substrate digestion step. *Lanes 6, 7:* Resting zone (lane 6) and growth zone (lane 7) chondrocyte matrix vesicles (40 μg protein) extracted with guanidine and dialyzed prior to electrophoresis. *Lanes 8, 9:* Resting zone (lane 8) and growth zone (lane 9) chondrocyte matrix vesicles (40 μg protein) extracted with guanidine, dialyzed, and incubated with 1 mM APMA prior to electrophoresis and during the substrate digestion step. **B:** *Lane 1:* Molecular weight markers. *Lanes 2, 3:* Resting zone (lane 2) and growth zone (lane 3) chondrocyte matrix vesicles (20 μg protein) extracted with guanidine containing PMSF, dialyzed, electrophoresed, and incubated in the presence of 10 ng/ml α_2-antiplasmin during substrate digestion. *Lanes 4, 5:* Resting zone (lane 4) and growth zone (lane 5) chondrocyte matrix vesicles extracted with guanidine containing PMSF, dialyzed, electrophoresed, and incubated in the presence of 100 μM AEBSF during substrate digestion. *Lanes 6, 7:* Resting zone (lane 6) and growth zone (lane 7) chondrocyte matrix vesicles extracted with guanidine containing PMSF, dialyzed, electrophoresed, and incubated in the presence of 4 mM 1,10-phenanthroline during substrate digestion. The approximate molecular weights of the active enzymes are shown on the right.

sin-1. This was confirmed by immunoprecipitating matrix vesicle and plasma membrane protein with anti-stromelysin-1 antibody, electrophoresis, Western blotting, and reprobing with antibody (Fig. 10). Staining was observed in the 48 and 29 kDa region for matrix vesicle samples (Fig. 10, lanes 1–4), while no immunoreactivity was detected in samples derived from plasma membrane (Fig. 10, lanes 5, 6) or media vesicle (data not shown) fractions. No additional immunoreactive bands were noted when the proteinase inhibitor, PMSF, was included in the sample prior to electrophoresis. However, increased staining was observed in those samples treated with PMSF, suggesting that the inhibitor blocked proteolytic degradation of the sample.

Detection of 72 kDa gelatinase in the membrane preparations required solubilization in PBS-TDS and freezing in the presence of PMSF. Matrix vesicles from cultures of resting zone and growth zone cells that were assayed for gelatinase activity without proteinase inhibitor produced zones of clearing in the 28–30 kDa region of the gel (Fig. 11A, lanes 4, 5). Zymograms of matrix vesicles that were stored in the presence of PMSF and assayed in the presence of α_2-antiplasmin contained additional bands at 72, 56, 50, and 33.5 kDa (Fig. 11A, lanes 2, 3). In contrast, the addition of 1,10-phenanthroline





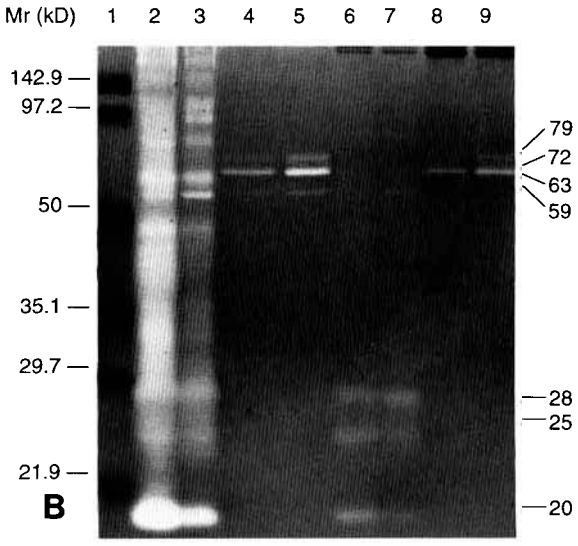


Fig. 10. Immunoblot of resting zone and growth zone chondrocyte matrix vesicles and plasma membranes immunoprecipitated with anti-stromelysin-1 antibody. Resting zone and growth zone chondrocyte matrix vesicles and plasma membranes were immunoprecipitated with anti-stromelysin-1 antibody, electrophoresed on 12.5% SDS-polyacrylamide gels, transferred to a PVDF membrane, and probed using the anti-stromelysin-1 antibody. All lanes contained 20 μg of protein. *Lane 1:* Resting zone chondrocyte matrix vesicles prepared in the presence of 0.5 mM PMSF. *Lane 2:* Growth zone chondrocyte matrix vesicles prepared in the presence of 0.5 mM PMSF. *Lane 3:* Resting zone chondrocyte matrix vesicles. *Lane 4:* Growth zone chondrocyte matrix vesicles. *Lane 5:* Resting zone chondrocyte plasma membranes. *Lane 6:* Growth zone chondrocyte plasma membranes. *Lane 7:* Normal sheep serum control.

during substrate digestion, after storage in PMSF, greatly reduced the amount of gelatinase activity found on the gel (Fig. 11A, lanes 6, 7), suggesting that the bulk of the gelatinolytic activity was due to metalloproteinases. Unlike the numerous enzymes in matrix vesicles that digest gelatin when PMSF is present (Fig. 11B, lanes 2, 3), media vesicles, with or without PMSF, only showed activity in three distinct regions (79, 72, and 59 kDa) of the gel (Fig. 11B, lanes 4, 5). Of these three bands, the 79 and 72 kDa bands were immunoprecipitable with anti-72 kDa gelatinase antibody (Fig. 11B, lanes 8, 9). In contrast, matrix vesicle proteins of M_r 28, 25, and 20 kDa were precipitable with the antibody and moderately active on gelatin after electrophoresis (Fig. 11B, lanes 6, 7); a fourth band of 72 kDa was also found, but its activity was weak. Western blots of media vesicle proteins from both resting zone and growth zone chondrocyte cultures demonstrated immunoreactivity with anti-72 kDa gelatinase antibody at M_r 72 kDa, while matrix vesicles and plasma membranes from both types of cultures were negative (data not shown).

In Vitro Phosphorylation of Stromelysin-1 by PKC

Recombinant human stromelysin-1 (rh-stromelysin-1) was observed as a 52 kDa band, while phosphorylated rh-stromelysin-1 was demonstrated as a 72 kDa band which demonstrated retarded mobility on SDS-PAGE (Fig. 12). The 52 kDa rh-stromelysin-1 band was present in the membranes probed with anti-stromelysin-1 antibody; rh-stromelysin-1 did not autophosphorylate itself (data not shown).

DISCUSSION

The results of the present study show that both resting zone and growth zone chondrocytes constitutively produce matrix metalloproteinases. The genes for stromelysin-1 and 72 kDa

Fig. 11. Gelatin zymography of resting zone and growth zone chondrocyte matrix vesicles and media vesicles. Resting zone and growth zone chondrocyte matrix vesicles and media vesicles (20 μg protein) were electrophoresed on 12.5% SDS-polyacrylamide gels containing 1 mg/mL gelatin. The presence of MMPs was determined by incubating the gels in 50 mM Tris buffer, pH 7.6, containing 5 mM $CaCl_2$ for 36 h at 37°C after removal of the SDS. After incubation, the gel was stained using Coomassie blue. Clear bands indicate the presence of proteolytic enzymes. **A:** *Lane 1:* Molecular weight markers. *Lanes 2, 3:* Resting zone (lane 2) and growth zone (lane 3) chondrocyte matrix vesicles were stored in the presence of PMSF and then incubated with 10 ng/ml α_2-antiplasmin during substrate digestion. *Lanes 4, 5:* Resting zone (lane 4) and growth zone (lane 5) chondrocyte matrix vesicles stored without PMSF. *Lanes 6, 7:* Resting zone (lane 6) and growth zone (lane 7) chondrocyte matrix vesicles stored in PMSF and then incubated with 4 mM 1,10-phenanthroline during substrate digestion. **B:** *Lane 1:* Molecular weight markers. *Lanes 2, 3:* Resting zone (lane 2) and growth zone (lane 3) chondrocyte matrix vesicles stored in PMSF. *Lanes 4, 5:* Resting zone (lane 4) and growth zone (lane 5) chondrocyte media vesicles. *Lanes 6, 7:* Resting zone (lane 6) and growth zone (lane 7) chondrocyte matrix vesicles stored in PMSF and immunoprecipitated using anti-72 kDa gelatinase antibody prior to electrophoresis. *Lanes 8, 9:* Resting zone (lane 8) and growth zone (lane 9) chondrocyte media vesicles immunoprecipitated using anti-72 kDa gelatinase antibody prior to electrophoresis.

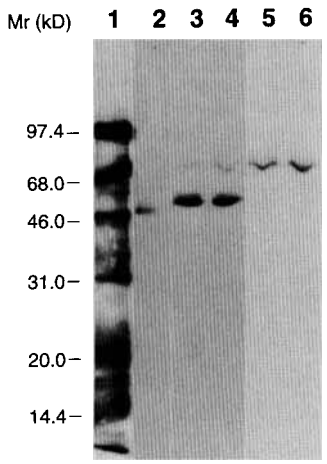


Fig. 12. In vitro phosphorylation of rh-stromelysin-1 by PKC. *Lane 1:* Molecular weight markers. *Lane 2:* Immunoblot of stromelysin-1 probed with anti-stromelysin-1; *Lanes 3, 4:* Immunoblot of stromelysin-1 phosphorylated by PKC (1 U PKC (lane 3) and 2 U PKC [lane 4]) demonstrating presence of a 50 kDa band as well as faint bands at 72 kDa; *Lanes 5, 6:* Autoradiogram of phosphorylated stromelysin-1 demonstrating retarded mobility on SDS-PAGE and the presence of 72 kDa band (1 U PKC (lane 5) and 2 U PKC [lane 6]).

gelatinase were expressed in both cell types. Further, resting zone chondrocytes had higher levels of mRNA for stromelysin-1 and 72 kDa gelatinase than growth zone cells. Although these differences were small, they were statistically significant. Immunohistochemistry also demonstrated the constitutive synthesis of the two matrix metalloproteinases, but this technique is not quantitative, and thus it is impossible for us to determine which cell type expressed more of each MMP.

In addition to the matrix metalloproteinases, TIMP-1 and TIMP-2 mRNAs were also expressed in these cultures. Although human probes were used for determining the gene expression of both TIMPs, the RT-PCR amplification products we obtained exhibited >92% sequence homology with sequences reported for human and mouse [Carmichael et al., 1986; Docherty et al., 1985].

Little is known about the specific localization of matrix metalloproteinases in the extracellular matrix. Our results indicate that the MMPs are differentially distributed within the culture, particularly within two extracellular membrane-bound compartments, matrix vesicles, and media vesicles. Based on casein zymography and Western blotting, stromelysin-1 was only found in matrix vesicles. In contrast, 72 kDa gelatinase was primarily localized to media vesicles. Matrix vesicles did exhibit weak gelatinase activity, but Western blots failed to detect immunoreactive 72 kDa protein. Neither stromelysin-1 nor 72 kDa gelatinase was observed in plasma membrane preparations, which is presumably the parent membrane of both matrix vesicles and media vesicles. This supports the hypothesis that matrix vesicles and media vesicles produced by resting zone and growth zone chondrocytes are discrete organelles capable of degrading various components of the extracellular matrix.

These data confirm our previous observations that matrix vesicles contain metalloproteinase activity [Dean et al., 1992]. In our original study, however, we demonstrated the presence of both neutral and acid metalloproteinase activities in plasma membranes preparations [Dean et al., 1992]. Failure to detect stromelysin or 72 kDa gelatinase activities in this membrane fraction in the present study suggests that other metalloproteinases are responsible. It is also possible that zymography, which is less sensitive than the radiolabeled proteoglycan degradation assay used previously, failed to detect the presence of metalloproteinases in the plasma membranes.

Differences in sensitivity in the two assays may also explain the lack of a quantitative difference in absolute enzyme activity between membrane fractions isolated from resting zone and

growth zone chondrocyte cultures. In the earlier study, we noted greater acid and neutral metalloproteinase activity in membrane fractions from growth zone cell cultures. In the present study, resting zone cell cultures exhibited lighter histochemical staining with the anti-stromelysin-1 antibody, suggesting a potential quantitative difference for this enzyme at least. However, zymography did not provide additional corroboration for this contention.

Matrix metalloproteinase activity in chondrocyte conditioned media is largely membrane-associated, since supernatants from culture media that had been centrifuged at 100,000*g* for 1 h contained little or no activity. The membranes present in the conditioned media were originally presumed to be fragments of plasma membrane because of their appearance in transmission electron micrographs and the fact that alkaline phosphatase activity is comparable to that of the plasma membrane [Boyan et al., 1988a]. Subsequent studies have demonstrated that the relative specific activities of a number of enzymes differ from not only those of the plasma membrane but of matrix vesicles as well [Boyan et al., 1988a]; the phospholipid metabolism of these membrane fractions is distinct [Schwartz et al., 1990], and they are regulated differentially by vitamin D metabolites [Boyan et al., 1988a]. Our results support the hypothesis that the media vesicles represent a distinct membrane fraction and suggest that they may serve as a mechanism for secretion of matrix metalloproteinases like gelatinase. How they might eventually be incorporated into the extracellular matrix, if at all, is unknown.

Our earlier studies suggested that most of the metalloproteinase activity in matrix vesicles was present in a fully activated form [Dean et al., 1992]. Our present results support this observation, although they do not definitively confirm it. The addition of APMA to membranes or membrane extracts prior to electrophoresis had little or no effect on the amount of activity observed or the migration of the MMP activity in the substrate gels, indicating that stromelysin-1 and 72 kDa gelatinase are present in matrix vesicles in active form. Whereas we had earlier found that it was necessary to first extract the enzymes to detect activity [Dean et al., 1992], no difference was found between native matrix vesicles or guanidine-HCl extracts when using zymography. This may have been due to the presence of SDS in the sample buffer and during electrophoresis, resulting in solubilization of the membrane, as well as partial unfolding of the protein, allowing it to digest the substrate.

It is also possible that other proteinases present in the membrane fractions activated latent stromelysin-1 and 72 kDa gelatinase after integrity of the matrix vesicle was lost. The higher molecular weight forms of the MMPs, as well as their enzyme activities, were preserved if the membranes were harvested in the presence of PMSF, solubilized in PBS-TDS, and then stored at −70°C. This suggested that inclusion of the proteinase inhibitor, PMSF, protected the MMPs from degradation by serine proteases. Although it may be argued that PMSF has a limited half-life, all membrane homogenizations and centrifugations were carried out at 4°C. There was virtually no occasion in the preparation of membrane fractions that the samples were stored at room temperature. It was difficult to exclude the possibility that after trypsinization of the cultures to release the matrix vesicles that some traces of trypsin remained in the samples and that this was the cause of the decrease in molecular weight and activity of the MMPs. That this was not the case is suggested by the fact that the activity of the ectoenzyme alkaline phosphatase remains high following matrix vesicle isolation [Boyan et al., 1988a,b; Majeska and Wuthier, 1975; Schwartz et al., 1989].

The results demonstrate that the 28–31 kDa forms of stromelysin-1 and, to a much smaller extent, 72 kDa gelatinase have significant levels of enzyme activity on the zymograms. Other studies have shown activity on zymograms in this molecular weight range for both of these MMPs [Nagase and Woessner, 1993]. Further, MMP-7 (matrilysin) [Crabbe et al., 1992; Woessner and Taplin, 1988] has apparent molecular weights of 28 kDa (latent) and 19 kDa (active) when assayed with zymography, and the presence of this MMP has not been examined by the present studies. Western analysis and inhibition of activity on substrate gels by 1,10-phenanthroline and lack of inhibition with AEBSF and α_2-antiplasmin strongly support the conclusion that the observed activity is due to the presence of metalloproteinases. The multiple bands seen on gelatin zymography may be due to the action of metalloproteinases other than gelatinase since

collagenase as well as stromelysin have been shown to degrade gelatin [Brenner et al., 1989].

Proteoglycan degradation is believed to be a major event in matrix remodelling prior to mineralization [Buckwalter et al., 1987; Kawabe et al., 1986; Landis et al., 1977] and includes degradation of proteoglycan aggregates and glycosaminoglycan chains [Poole et al., 1982, 1989]. Cytochemical studies have demonstrated that glycosaminoglycan degradation is mediated by matrix vesicles [Takagi et al., 1981], and other studies have localized stromelysin-1 and collagenase to the hypertrophic cell zone of the growth plate, the region where mineralization occurs [Brown et al., 1989; Dean et al., 1985]. Since MMPs are present in matrix vesicles in active form [Dean et al., 1992], it is likely that breakdown of the matrix vesicle membrane is a key regulatory event in mineralization.

It is intriguing to hypothesize that matrix vesicles may enable cells to control events in the extracellular matrix at sites distant from the cell membrane [Boyan et al., 1994]. Evidence in support of this hypothesis includes the nongenomic regulation of matrix vesicle phospholipase A_2 activity by vitamin D metabolites [Schwartz et al., 1989], resulting in the loss of membrane integrity due to the hydrolysis of phospholipids, as well as the recent observation that regulation of matrix vesicle metalloproteinase activity by β-glycerophosphate and ascorbic acid correlates with formation of mineral in cultures of osteoblast-like cells [Dean et al., 1994].

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