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Secretion of Matrix Metalloproteinases and Their Inhibitors (Tissue Inhibitor of Metalloproteinases) by Human Prostate in Explant Cultures: Reduced Tissue Inhibitor of Metalloproteinase Secretion by Malignant Tissues[1]

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ABSTRACT

Unregulated secretion of matrix metalloproteinases (MMPs) or their endogenous protein inhibitors (tissue inhibitor of metalloproteinases, TIMPs) has been implicated in tumor invasion and metastasis. Species of MMPs and TIMPs secreted by epithelial cultures of normal, benign, and malignant prostate were identified and their levels were compared. Fragments of fresh tissue were cultured in a serum-free medium that supported the outgrowth of prostatic epithelial cells. Biochemical analysis of the conditioned media by gelatin zymography and enzyme assays showed that both normal and neoplastic tissues secreted latent and active forms of both M_r 72,000 type IV collagenase (MMP-2) and M_r 92,000 gelatinase (MMP-9). However, conditioned media from malignant prostate explants contained a higher proportion of the active form of MMP-2. Significant amounts of free TIMPs were secreted by normal juvenile and adult prostates, but they were either markedly reduced or not detectable in conditioned media from neoplastic tissues. These findings suggest that there is an imbalance of secretion between MMPs and TIMPs in prostatic carcinoma.

INTRODUCTION

Metastasis of an initially localized tumor to vital organs is the dominant cause of cancer related deaths (1). The mechanism(s) controlling the metastatic progression of a localized tumor is a very complex process, involving many biochemical and cellular events (2). One such biochemical event may be the secretion of proteolytic enzymes, capable of degrading the extracellular matrix by invading tumor cells. Penetration of the basement membrane surrounding the tumor by cells that can secrete and locally initiate a proteolytic cascade is the first step in tumor invasion. The invading cell must then penetrate and degrade the basement membrane underlying the vascular endothelium to enter circulation (3). Following its arrest in the target organ, the tumor cell(s) must "reinvade" through the vascular endothelium and enter the stroma to colonize the new tissue.

Degradation of basement membrane, which is mainly composed of type IV collagen, laminin, and fibronectin, is mediated by a set of secreted MMPs[3] also termed matrixins (4). These naturally occurring, Zn^{2+}-dependent, endopeptidases are involved in the normal turnover of connective tissue matrix, as well as in certain disease processes (5). These matrixins are MMP-2, an M_r 72,000 collagenase that digests collagen types IV, V, VII, and X, elastin, fibronectin, and gelatins; MMP-9, an M_r 92,000 gelatinase that digests collagens IV and V and gelatins; and MMP-3 (stromelysin), which degrades collagens III, IV, V, and IX, as well as the noncollagenous components of the extracellular matrix, including laminin and fibronectin (6). In addition, MMP-3 is also known to activate procollagenases (7). Increased levels of these proteinases have been implicated with the invasive potential of tumors (8–10).

Matrixins secreted by normal cells are proenzymes (zymogens) and are inactive (11). This is due to a highly conserved sequence of nine amino acid residues in the propeptide region containing a solitary cysteine residue bound to the metal ion, Zn^{2+}, at the active center (12). The enzymes are activated when a prosegment peptide is pulled away from the active center by breaking the cysteine-zinc contact and cleaved from the proenzyme (13). This is thought to occur *in vivo* by proteolysis and also can be accomplished *in vitro* by using trypsin, plasmin, or organomercurial compounds (*e.g.*, APMA). Treating latent MMPs with APMA results in active-intermediate forms of reduced molecular mass. Autocatalytic cleavage of the prosegment results in permanent activation of the enzymes (5). Ionic detergents such as SDS also activate these enzymes without cleaving the prosegment.

Proteolysis by MMPs is regulated by a family of naturally occurring endogenous proteinase inhibitors known as TIMPs (14). There are at least two molecular species of TIMPs: TIMP-1 (M_r 28,000) and TIMP-2 (M_r 21,000) (15, 16), as well as a large TIMP-like inhibitor (M_r 76,000) (17). Many cell lines secrete MMP-2 and MMP-9 as proenzyme-inhibitor complexes with TIMPs (18–20).

Although both normal and neoplastic cells produce MMPs and other proteinases (21, 22), only malignant cells are invasive (23). Therefore, it is more likely that control of MMP activity by specific inhibitors (*e.g.*, TIMPs) is a cause for the differential functioning of these enzymes in normal and neoplastic tissues. For example, uncontrolled secretion or constitutive activation of secreted MMPs with a concomitant decrease in TIMP production might be responsible for the invasive property of some prostate tumor cells. We tested this hypothesis by measuring the pattern of secretion of both MMPs and TIMPs in tissue explants of normal, benign (BPH), and malignant prostate. These were cultured in a serum-free medium that supported the outgrowth of epithelial cells. Our findings suggest that, although the epithelial cultures of both normal and neoplastic prostate secrete MMP-2 and MMP-9, it is the pattern of secretion of TIMPs that clearly distinguishes between benign and malignant neoplasms in the prostate.

MATERIALS AND METHODS

Explant Cultures. Normal prostatic tissues, from adults (28–35 years) and juveniles (4–12 years), were obtained from organ donors. Tissue procurement from donors was performed according to state and federal regulations. Neoplastic tissues as chips were obtained from patients undergoing open prostatectomy for BPH or for PC (51–72 years). Prostatic specimens, from 4 adults, 5 juveniles, 10 patients with BPH, and 6 patients with PC, collected over 2 years, were analyzed for this study. The tumor grades of all PC samples analyzed by the University of Miami Pathology Reference Service were between 4 and 6 [according to the classification of Gleason (24)].

Fresh tissue (1–5 g), explanted under sterile conditions, was processed within 1–3 h after surgery. Tissues were minced into small pieces (~10 mm^3), rinsed several times in sterile 10 mM phosphate buffer containing 150 mM

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[3] The abbreviations used are: MMPs, matrix metalloproteinases; APMA, *p*-aminophenylmercuric acetate; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; TIMPs, tissue inhibitors of metalloproteinases; BPH, benign prostatic hyperplasia; PC, prostate cancer; CM, conditioned medium (media); UMP, metalloproteinase from rat uterus.

NaCl, and seeded in T25 Falcon Primaria flasks (~100 mg tissue/flask). Tissue fragments were cultured in MEGM, a serum-free epithelial growth medium (Clonetics Corp., San Diego, CA). MEGM is a modified MCDB 170 medium (25) supplemented with epidermal growth factor (10 ng/ml), insulin (0.5 μg/ml), hydrocortisone (0.5 μg/ml), bovine pituitary extract (0.4%), gentamicin sulfate (0.1 mg/ml), and amphotericin B (1000 IU/ml). Medium covering the explants was renewed after the first 24 h of culture. CM was then collected and exchanged with fresh medium weekly for 8–10 weeks. Cells, epithelial in morphological appearance, grew out of explant tissue but did not cover the entire flask during the culture period. The epithelial nature of the cell monolayer was confirmed by immunocytochemical staining, using an anti-cytokeratin monoclonal antibody specific to cytokeratins 8 and 18 (CAM 5.2; Becton-Dickinson Immunocytometry Systems, San Jose, CA). Greater than 90% of the cells stained positive with CAM 5.2 in five randomly selected microscopic fields under observation. The percentage of cytokeratin-positive cells in a given area was independent of the type of the explant tissue. The secretion of prostate-specific antigen into the culture medium was confirmed by immunoblotting with a commercial antibody to prostate-specific antigen (Sigma Chemical Co., St. Louis, MO).

Partial Purification of MMPs and TIMPs. Conditioned media (5 ml) from various explant cultures were applied directly to a calibrated Ultrogel AcA-54 column (1.6 x 81 cm) equilibrated with 0.05 M Tris-HCl, pH 7.5, containing 0.01 M $CaCl_2$, 0.2 M NaCl, and 0.005% Brij 35. Fractions (4.3 ml) were collected and assayed for total proteins, MMPs, and TIMPs. Proteins in the serum-free conditioned culture media were assayed by the method of Bradford (26).

Assays for MMPs. Column fractions were assayed for various MMPs using: (*a*) ^{3}H-acetylated type 1 gelatin to estimate the MMP-2 and MMP-9 gelatinases (27), (*b*) ^{3}H-acetylated type 1 rat skin collagen for MMP-1 (interstitial collagenase) by the method of Dean and Woessner (28), and (*c*) ^{3}H-acetylated proteoglycan monomer bead assay for stromelysin (MMP-3), as described by Gunja-Smith *et al.* (29). Enzyme assays were carried out with or without addition of 1–1.5 mM APMA for estimating latent and/or active MMPs. Blanks were obtained by adding 1–1.5 mM 1,10-phenanthroline.

Assays for Inhibitors of MMPs. Activity of TIMPs in column fractions was measured by the inhibition of a partially purified small UMP (M_r 19,000) in the same manner as reported by Zhu and Woessner (30) using Azocoll as a substrate (Calbiochem Corp., La Jolla, CA). Briefly, fractions from the Ultrogel AcA-54 column (10–250 μl) were preincubated with UMP (15–20 ng/400 μl) for 1 h at 37°C. After the Azocoll suspension (2.0 mg/850 μl) was added, tubes were incubated for 18 h at 37°C. Assay blanks were set up with substrate only and with 1,10-phenanthroline. After undigested Azocoll was centrifuged, the absorbance of the supernatant was measured in a spectrophotometer at 520 nm. Presence of inhibitor (TIMP) in the column fractions was detected by the decrease in A_{520} as a result of enzyme inhibition. One mol of uterine MMP was assumed to bind with 1 mol of TIMP, as reported previously (30).

Gelatin Zymography. The gelatinases (MMP-2 and MMP-9) in various conditioned media were further analyzed by a gelatin-zymography technique, using a slightly modified procedure from that of Herron *et al.* (31). Briefly, the proteins from column fractions or conditioned media were separated by SDS-PAGE (32) using 10% acrylamide copolymerized with gelatin (0.33 mg/ml) (33). After electrophoresis, the gels were rinsed twice in 0.25% Triton X-100 for 20 min, at 21°C, and then incubated for 18 h at 37°C in substrate buffer (0.05 M Tris-HCl (pH 7.5), 0.2 M NaCl, 0.01 M $CaCl_2$, 1 μM $ZnCl_2$, 1 mM phenylmethylsulfonyl fluoride, 0.02% NaN_3, and 0.005% Brij 35). Gelatinases were identified following staining of the gel in 0.25% Coomassie blue R250 in 40% 2-propanol (1 h) and destaining in 7% acetic acid. The digested area appeared clear on a blue background indicating the location of gelatinases. Although MMP-2 migrates at M_r 72,000 under reducing conditions, it migrated at M_r 68,000 under the nonreducing substrate gel conditions (34). Similarly, APMA-activated MMP-2 migrated at M_r 60,000. For consistency with other published reports, we have labeled the latent and activated MMPs according to their deduced M_r (5) in Figs. 3, 4, and 5.

Reverse Zymography. TIMP-1 and TIMP-2 secreted into the culture medium were detected using a modification of the reverse zymography method of Herron *et al.* (31). Briefly, 10 μl of 10-fold concentrated conditioned media from various explant cultures were fractionated by SDS-PAGE. A 12% polyacrylamide-gelatin (0.75 mg/ml) gel was used. The gel was rinsed twice in 50 mM Tris-HCl-0.1% Triton X-100 solution (20 min each) and incubated for 1–3 h at 37°C with a mixture containing the substrate buffer, APMA-activated MMP-2 (1–2 units/ml, purified from human skin fibroblasts), and the rat uterine collagenase (MMP-1, 0.5 units/ml). The enzyme mixture was then removed, and the gel was incubated for 16–18 h with only substrate buffer at 21°C. Undigested gelatin in the gel was visualized by staining in 0.1% followed by 0.25% Coomassie blue and destaining in 7% acetic acid. Areas of the gel containing TIMPs appeared as dark blue bands against a paler blue background.

RESULTS

Characterization and Estimation of Secreted MMPs. Conditioned media from epithelial outgrowth of prostate tissue explants were tested for collagenase and gelatinase activity. Initial attempts to digest ^{3}H-acetylated gelatin or ^{3}H-acetylated type 1 collagen using whole conditioned media from nonmalignant tissues were unproductive. We suspected the presence of inhibitors, such as TIMPs, in the culture media. Therefore, 5 ml of CM was fractionated by molecular sieve chromatography on an Ultrogel AcA-54 column to separate the enzymes from the inhibitors. A clear separation of enzyme activity from that of inhibitors was achieved when the flow rate was adjusted to 11–12 ml/h. A chromatogram of the CM collected from the normal adult prostate explant is shown in Fig. 1. Fractions 16–18 contained significant amounts of gelatinase activity as determined by [^{3}H]gelatin digestion assay. Fractions 23–29 contained TIMP activity as determined by inhibition assays on Azocoll. Fractions of conditioned media from all prostatic primary cultures showed the presence of latent gelatinases which were assayed after activation with 1 mM APMA. However, significant amounts of active gelatinases were detected in the column fractions of conditioned media from malignant (40–50%) and juvenile (10–20%) prostate explants (Fig. 2). On the other hand, as shown in Fig. 2, only small amounts (0–5%) of active enzyme were detected in BPH conditioned media and none in normal adult prostate explants. In addition, significant amounts of TIMPs were also detected in column fractions of the conditioned media from normal juvenile and adult prostates and BPH tissue explants. However, neither type I collagenase (MMP-1) nor stromelysin (MMP-3) was detected in any of the column fractions from any prostate explant conditioned media when assayed using ^{3}H-acetylated rat skin collagen and by ^{3}H-acetylated proteoglycan monomer beads (28, 29).

Since gelatinase(s) was the only MMP detected in the column fractions, the fractions containing gelatinase activity were further

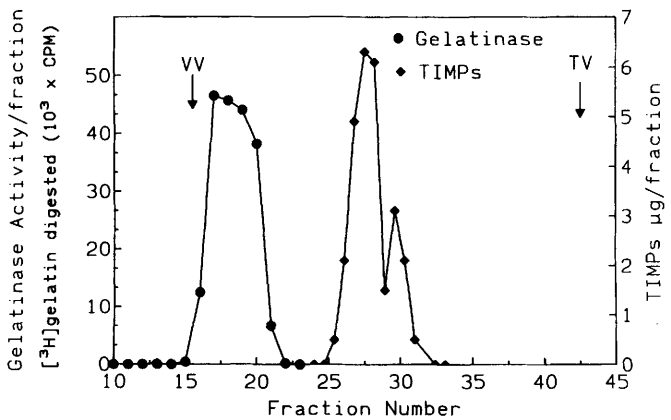

Fig. 1. Separation of gelatinases (MMPs) and TIMP activity by molecular sieve (Ultrogel AcA-54) chromatography of conditioned medium. CM (5 ml) from an explant culture of normal adult prostate tissue was separated on the column. Enzyme/inhibitor activities in the eluted fractions were assayed as described in "Materials and Methods." One unit of gelatinase activity = 1 μg [^{3}H]gelatin (8300 cpm) digested/min at 37°C. Analyses of other explant cultures also produced similar chromatographic profiles with different quantities of enzyme and inhibitor activities. VV, void volume (60 ml); TV, total elution volume (170 ml). Protein content of the individual fractions was below the detection limit (<10 μg/ml).

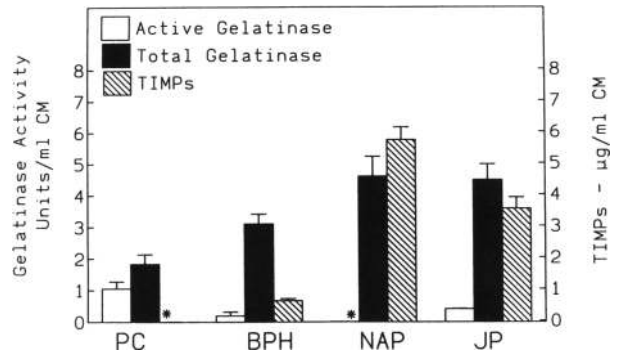

Fig. 2. Estimates of secreted MMPs and free-TIMPs. Gelatinases and TIMP activities were measured from respective pooled peak fractions following column chromatography. Enzyme and inhibitor activities are normalized to an equivalent volume of CM. Estimates of total and active enzyme activities were deduced from enzyme assays ± 1 mM APMA activation. One unit of gelatinase activity = 1 µg of [^{3}H]gelatin digested/min at 37°C. *Column* (*bar*), mean (±SEM) from four to six donor samples, measured in the conditioned media collected weekly for at least 4 weeks. PC, primary carcinoma of the prostate; NAP, normal adult prostate; JP, juvenile (prepubertal) prostate; *, <0.01 units/ml or <0.1 µg/ml. TIMPs were not detected (<0.1 µg/ml) in the PC-conditioned media, and activated gelatinases were not detected (<0.01 unit/ml) in the conditioned media from normal prostate explants.

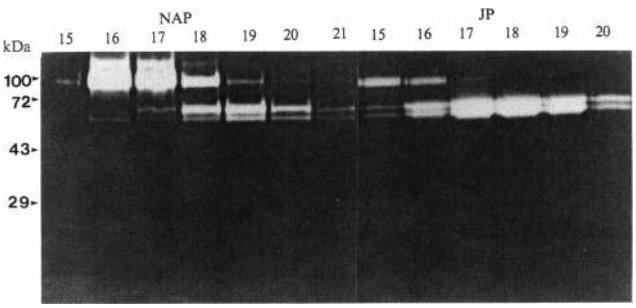

Fig. 3. Zymographic detection of species of gelatinases secreted by explant cultures. Since the total protein content of various column fractions or of the CM was below the detection limits in the protein assay (<10 µg/ml), a constant volume (20 µl) of column fractions of conditioned media from prostate explants (juvenile and normal adults) was separated by SDS-PAGE on a gelatin-embedded gel. Zymography was performed as described in "Materials and Methods." Localization of MMP-2 (M_r 72,000) and MMP-9 (M_r 92,000) relative to that of the molecular weight standards (prestained markers; BRL, Gaithersberg, MD) is shown. Under nonreducing conditions, latent and activated MMP-2 migrated at M_r 68,000 and 60,000, respectively. *Ordinate*, M_r (in thousands); *abscissa*, fraction number. The zymogram shown is for a single explant using culture medium collected at week 3. Similar results were obtained from analogous explants. NAP, adult prostate; JP, juvenile prostate.

analyzed by gelatin-zymography to identify the species of gelatinases present. As shown in Fig. 3, there were clear differences in the molecular species and amounts of gelatinases secreted by various prostatic specimens. Explant cultures of normal prostate (both juvenile and adult) secreted MMP-2 (M_r 72,000) and MMP-9 (M_r 92,000), but the relative amounts of each species and the proportion of active to latent enzyme was striking. Latent MMP-9 was the predominant species of gelatinase secreted by adult prostate explants, although a low amount of latent MMP-2 was also detected. The conditioned media from juvenile prostate tissue explants, on the other hand, contained low amounts of latent MMP-9 but a larger amount of MMP-2 composed of both active and latent forms. Because of the significant differences in the rates of gelatin digestion by MMP-9 and MMP-2 (35), no attempts were made to quantitate MMP-2 and MMP-9 resolved by zymography.

Characterization of MMPs Secreted by the Neoplastic Prostate Explants. Zymography of conditioned media from BPH and malignant tissue explants (Fig. 4) revealed differences in the ratio of active to latent forms of the MMPs and in the relative proportion of each molecular species secreted by the benign and malignant prostate tissue explants. Explants of benign neoplasms were found to secrete only the latent form of MMP-9 (M_r 92,000), but the MMP-2 secreted was a mixture of the two molecular forms (M_r 72,000 and 66,000, latent and active, respectively), with the zymogen predominating. Malignant prostate tissue explants (PC) also secreted latent MMP-9, like the BPH explants, but the ratio of active to latent MMP-2 was reversed in PC, with the active form of MMP-2 (M_r 66,000) as the major fraction.

As shown in Fig. 4, in addition to the latent MMP-9 and active MMP-2, a minor gelatinase band at M_r ~110,000 was also detected in BPH explant CM. BPH tissue explant CM was chromatographed on an AcA-54 column, and the fractions were incubated with 1 mM APMA to activate the gelatinases. The APMA-activated fractions along with control fractions were then analyzed by zymography to identify the various M_r forms of activatable gelatinases. APMA activation was also done to confirm that the higher M_r forms of these enzymes were indeed latent MMP-9 and MMP-2, respectively. APMA-activated enzymes migrated faster during SDS-PAGE, due to a reduction in their molecular weights and, therefore, localized below the latent forms in the zymogram. Fig. 5 is a typical zymogram showing control and APMA-activated MMPs, using conditioned media from a BPH explant. Similar analyses of conditioned media from other prostate tissue explants also showed that both MMP-2 and MMP-9 were activated when treated with APMA, and they were the only species of gelatinases present in the CM (data not shown).

Characterization and Estimation of Secreted TIMPS. Assays for inhibitory activity of conditioned media using the inhibition of UMP enzyme activity on Azocoll revealed significant differences in the amounts of inhibitor(s) expressed. Conditioned media from normal prostate tissues (both juvenile and adult) showed significant activity of MMP inhibitors. As summarized in Fig. 2, conditioned media from adult prostate explants had the highest levels of inhibitor activity (5.25 ± 0.40 µg/ml, mean ± SEM), followed by that from juvenile prostate explants (3.6 ± 0.35 µg/ml), with some activity in the conditioned media from the BPH explants (0.7 ± 0.07 µg/ml). Inhibitor activity was undetectable in PC explant samples (≤0.003 µg/ml).

The Azocoll assay system could detect only unbound (free) inhibitor(s) in the samples tested. The apparent lack of inhibitor(s) in malignant prostate explant cultures could also be due to enzyme-inhibitor

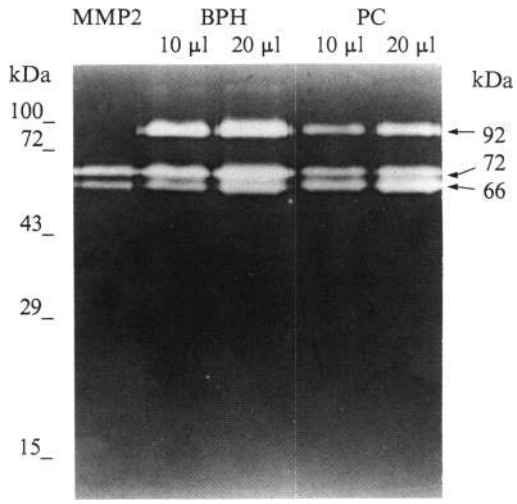

Fig. 4. Identification of secreted MMPs from conditioned media of neoplastic explants. Conditioned media (10 and 20 µl) were separated on a gelatin-embedded polyacrylamide gel by SDS-PAGE. *Lane 1*, MMP-2 purified from a serum-free medium conditioned by human skin fibroblasts. Zymography profiles of a single BPH and PC explant culture are shown. Similar profiles were obtained from seven independent BPH explants and three PC explants.

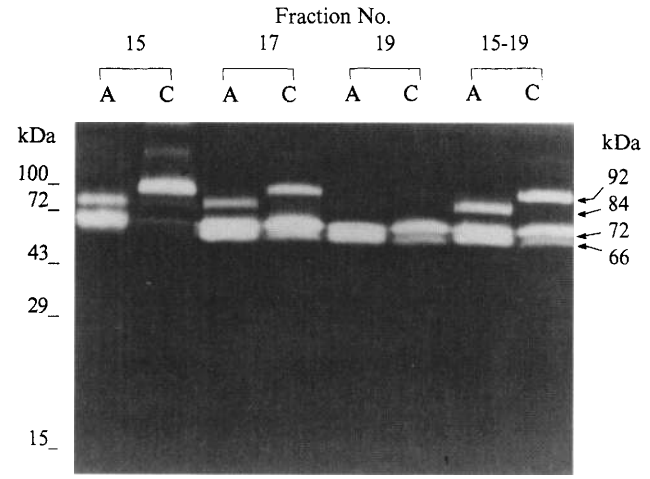

Fig. 5. Zymography of column fractions (AcA-54) of media conditioned by BPH explant culture with and without APMA activation. This figure shows M_r of activatable forms of gelatinases (MMP-2 and MMP-9) in column fractions. Column fraction 15, mainly MMP-9 (<92%); fraction 17, a mixture of MMP-2 and MMP-9; fraction 19, mainly (>90%) MMP-2; fractions 15–19, aliquot of combined fractions separated by gelatin-SDS-PAGE following a 2-h incubation ± 1 mM APMA. Digestion of gelatin in the gel by localized MMPs was allowed to proceed for 18 h at 37°C. *Ordinate,* M_r (in thousands) of gelatinases, latent and active. *Abscissa: A,* APMA activated; *C,* untreated samples.

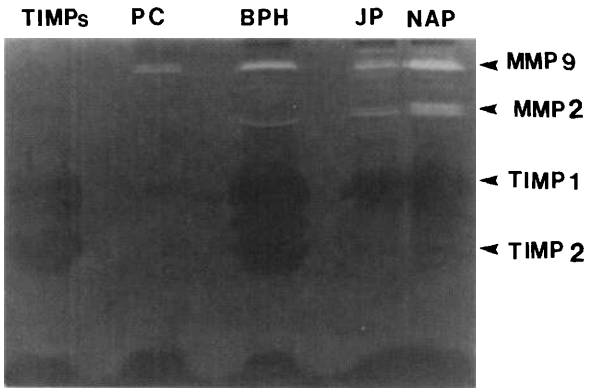

Fig. 6. Detection of TIMPs by reverse zymography: 10-fold concentrated CM from various explant cultures were analyzed. These were fractionated on a 12% polyacrylamide gel by SDS-PAGE. Gels contained gelatin (0.75 mg/ml) as the substrate. Reverse zymography was done as described in "Materials and Methods." *Arrowheads,* relative location of purified recombinant TIMP-1 and TIMP-2 (~125 ng each). *Lane 2,* CM from PC; *lane 3,* CM from BPH; *lane 4,* CM from normal adult prostate (NAP); *lane 5,* CM from juvenile prostate (JP).

complexes of MMP and TIMP (36). Therefore, samples of culture media were heated (60°C, 30 min) in the presence of 1 mM EDTA, pH 3.0, to dissociate MMP-TIMP complex and release free TIMP. However, assays for UMP inhibition using the method of Zu and Woessner (30) with heat-treated samples were still negative. It was also possible that the only inhibitor present in the conditioned media from malignant prostate explants was TIMP-2 which does not inhibit UMP efficiently in the present assay conditions (37). Therefore, reverse zymography was performed to detect the molecular species of the inhibitors present in various conditioned media (Fig. 6). BPH-conditioned media showed the presence of both an M_r 28,000 (TIMP-1) and 21,000 (TIMP-2) molecular species that prevented the digestion of gelatin in the gel. TIMP-1 was detected in the CM of normal adult and juvenile prostate and BPH tissue explants, but only a trace amount of TIMP-1 was detected in the CM of PC explants. In addition, TIMP-2 was also absent in the CM from both normal juvenile prostate and in PC tissue explants.

DISCUSSION

The present study clearly demonstrates that prostate epithelial cells secrete both latent and active forms of MMPs. Additionally, normal prostatic epithelia, as well as that derived from BPH tissue, secrete TIMPs. These findings strongly suggest that the basement membrane underlying prostatic epithelium probably undergoes rapid turnover due to the matrix-degrading enzymes secreted by the epithelium.

A novel finding of this study is that only two species of matrixins, MMP-2 and MMP-9, were found in the conditioned media. Other classes of MMPs such as interstitial type 1 collagenase (MMP-1), stromelysin (MMP-3), PUMP-1 (MMP-7), and transin-2 (MMP-10) were absent in the conditioned media analyzed from prostate with different neoplastic dispositions. Whether the secretion of a limited number of matrixins is due to a selective environment of the *in vitro* culture system is not clear at present. For example, we used an artificial substratum (plastic) and a serum-free medium that supported only epithelial cell outgrowth. Recently, Jones and Harper (38) expressed doubts about the suitability of an explant culture model as truly representing *in vivo* processes. Their studies of the morphological changes and cellular origin of epithelial cell outgrowth from BPH tissue explants showed that the epithelial cells were of basal cell origin, a rare cell population in intact tissue and an uncommon source of hyperplasia or malignancy. The culture conditions we used differed from theirs. Using a similar culture conditions, we were able to identify the expression of cytokeratin types 8 and 18,[4] a characteristic of glandular prostatic epithelium in intact tissue (39). Furthermore, using the same culture conditions, we found not only different proportions of active and latent MMPs but also different levels of MMPs and TIMPs, depending on the source of the tissues. These results correlate remarkably with the pathological state of these tissues *in vivo*. Studies in which intact prostatic tissue was used were reported earlier (40, 41). However, our studies using isolated epithelial cultures have the advantage of detecting the dynamics of enzyme and inhibitor secretion by malignant or normal prostatic epithelium. Furthermore, these studies in the future might also help to understand the factor(s) that influence the secretion of MMPs by the prostatic epithelia.

Pajouh *et al.* (40) reported the presence of mRNA for MMP-2 and MMP-7 in malignant prostate by Northern blotting, and they localized these messages to the epithelial compartment by the *in situ* hybridization technique. Recently, Stearns and Wang (41) showed the presence of transcripts for MMP-2 and immunoreactivity for anti-MMP-2 antibody in malignant prostate. They also reported a higher fraction of activated MMP-2 in malignant prostate tissue compared with that in BPH. We extend some of their findings, although at the level of protein expression, to all types of prostate tissues. Furthermore, results presented here show the secretion of activated forms of MMPs by only juvenile or malignant tissues (Fig. 2) but not normal adult prostate explants. This probably indicates that both of the tissues, juvenile (prepubertal) and malignant, undergo rapid changes in their basement membrane architecture, the former as a result of normal prepubertal growth and the latter, presumably, the onset of an invasive phenotype. However, a significant difference between the conditioned media from juvenile and malignant tissue explants was that the former also contained significantly high amounts of TIMPs, a plausible requirement for controlling or arresting the activity of MMPs.

Using two different assays, we found an apparent disparity in the estimates of active MMP-2 secreted by BPH tissue explants. Only a small amount of active MMPs was detected in the biochemical assay (Fig. 2), but zymography revealed a substantial amount of active

[4] H. Matzkin, B. L. Lokeshwar, N. L, Block, and M. S. Soloway, Human prostatic cytochrome P450 aromatase: demonstration of its existence in human prostate cells, manuscript submitted.

MMP-2 in BPH explant-conditioned media (Fig. 4). We offer the following rationale as a plausible cause of this anomaly. Clark *et al.* (42) showed that, when latent forms of MMPs are activated in the presence of TIMP, they form an inactive enzyme-inhibitor complex. When formed, the complex of active enzyme and TIMP cannot be dissociated to yield TIMP-free active enzyme; conditions used to dissociate the complex (36) destroy the enzyme activity, while the inhibitor activity is still retained. Therefore, it is plausible that we obtained a lower estimate of active enzymes in the conditioned media of BPH explants using the gelatinase assay.

We found continued secretion of MMPs and, in normal cases, also TIMPs in explant cultures. This pattern of secretion was stable for the entire culture period without any significant change in the proportion of active to latent forms.

Type IV collagen and laminin are the most abundant of the basement membrane structural proteins in normal prostate tissue. Barskey *et al.* (43) and recently Sinha *et al.* (44) reported extensive studies of basement membrane composition and their selective degeneration in the various stages of prostatic carcinoma. Several *in vitro* studies in which metastatic adenocarcinoma cell lines of the colon (45), breast (22), and prostate (46) were used also suggested that degradation of the components, type IV collagen and laminin, in basement membrane is the single most significant step in the invasion and metastasis of these tumor cells.

Clearly, the secretion of active MMP-2 is not unique to tumor cells because prepubertal prostate cells also secrete active MMP-2. Therefore, secretion of active MMP-2 alone is not sufficient for uncontrolled degradation of basement membrane. Furthermore, secretion of nonmetalloproteinases, such as the urokinase-like proteinases, might also significantly contribute to this pathogenesis (47). There may be many factors responsible for the degradation of basement membrane in prostatic malignancy. But, as Liotta *et al.* (48) proposed, it may also be a result of the "imbalance" between the negative and positive factors. One such negative invasion factor might be TIMP. Normal cells presumably secrete "balanced" amounts of TIMPs and MMPs so that there is a controlled turnover of the basement membrane. However, as we have shown here, malignant cells secrete predominantly active MMPs and little, if any, TIMP. Inhibiting the activation of latent MMPs, supplementing TIMP, or activating the secretion of TIMP might, therefore, reduce the incidence of invasion and metastasis as shown recently (49–51).

More than one mechanism may be involved in regulating basement membrane turnover during normal and neoplastic growth of prostatic epithelia. The results presented here provide evidence for a possible intrinsic control mechanism involving combined secretion of MMPs and their inhibitors for basement membrane turnover.

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REFERENCES

1. Liotta, L. A., and Stetler-Stevenson, W. G. Metalloproteinases and cancer invasion. Semin. Cancer Biol., *1:* 99–106, 1990.
2. Zetter, B. R. The cellular basis of site-specific tumor metastasis. N. Engl. J. Med., *322:* 605–612, 1990.
3. Liotta, L. A. Tumor invasion and metastases: role of the basement membrane. Am. J. Pathol., *117:* 339–348, 1984.
4. Moscatelli, D., and Rifkin, D. B. Membrane and matrix localization of proteinases: a common theme in tumor cell invasion and angiogenesis. Biochim. Biophys. Acta, *948:* 67–85, 1988.
5. Woessner, F. J., Jr. Matrix metalloproteinases and their inhibitors in connective tissue remodeling. FASEB J., *5:* 2145–2154, 1991.
6. Emonard, H., and Grimaud, J-A. Matrix metalloproteinases: a review. Cell. Mol. Biol., *36:* 131–153, 1990.
7. Suzuki, K., Enghild, J. J., Morodomi, T., Salvesen, G., and Nagase, H. The mechanisms of activation of tissue procollagenase by matrix metalloproteinases (stromelysin). Biochemistry, *29:* 10261–10270, 1990.
8. Liotta, L., Tryggvason, K., Gabrisa, S., Hart, I., Foltz, C. M., and Shafie, S. Metastatic potential correlates With enzymatic degradation of basement membrane collagen. Nature (Lond.), *284:* 67–69, 1980.
9. Zucker, S., Beck, G., DiStefano, J. F., and Lysik, R. M. Role for different cell proteinases in cancer invasion and cytolysis. Br. J. Cancer, *52:* 223–232, 1985.
10. Ura, H., Bonfil, R. D., Reich, R., Reddel, R., Pfeifer, A., Harris, C. C., and Klein, A. J. P. Expression of type IV collagenase and procollagen genes and its correlation with the tumorigenic, invasive and metastatic abilities of oncogenic transformed human bronchial epithelial cells. Cancer Res., *49:* 4615–4621, 1989.
11. Okada, Y., Harris, E. D., Jr., and Nagase, H. The precursor of a metalloendopeptidase from human rheumatoid synovial fibroblasts: purification and mechanisms of activation by endopeptidases and 4-aminophenylmercuric acetate. Biochem. J., *254:* 731–741, 1988.
12. Vallee, B. L., and Auld, D. S. Zinc coordination, function and structure of zinc enzymes and other proteins. Biochemistry, *29:* 5647–5649, 1990.
13. Springman, E. B., Angleton, E. L., Burkedal-Hansen, H., and Van Wart, H. E. Multiple modes of activation of latent human fibroblast collagenase: evidence for the role of a Cys^{73} active-site zinc complex in latency and a "cysteine switch" mechanism for activation. Proc. Natl. Acad. Sci. USA, *87:* 364–368, 1990.
14. Cawston, T. E. Protein inhibitors of metalloproteinases. *In:* A. J. Barrett and G. Salvesen (eds.), Proteinase Inhibitors, pp. 589–610. Amsterdam, The Netherlands: Elsevier, 1986.
15. Boone, T. C., Johnnson, M. J., De Clerck, Y. A., and Langley, K. E. cDNA cloning and expression of a metalloproteinase inhibitor related to tissue inhibitor of metalloproteinases. Proc. Natl. Acad. Sci. USA, *87:* 2800–2804, 1990.
16. Stetler-Stevenson, W. G., Krutsch, S. C., and Liotta, L. A. Tissue inhibitor of metalloproteinase (TIMP-2). A new member of the metalloproteinase inhibitor family. J. Biol. Chem., *264:* 17374–17378, 1989.
17. Cawston, T. E., Curry, V. A., Clark, I. M., and Hazelman, B. L. Identification of a new metalloproteinase inhibitor that forms tight-binding complexes with collagenase. Biochem. J., *269:* 183–187, 1990.
18. Murphy, G., Cawston, T. E., Galloway, W. A., Barnes, M. J., Bunning, R. A. D., Mercer, E., Reynolds, J. J., and Burgeson, R. E. Metalloproteinases from rabbit bone culture medium degrade types IV and V collagens, laminins and fibronectin. Biochem. J., *199:* 807–811, 1981.
19. Umenishi, F., Umeda, M., and Miyazaki, K. Efficient purification of TIMP-2 from culture medium conditioned by human hepatoma cell line, and its inhibitory effects on metalloproteinases and *in vitro* tumor invasion. J. Biochem., *110:* 189–195, 1991.
20. Wright, J. K., Clark, I. M., Cawston, T. E., and Hazelman, B. L. The secretion of the tissue inhibitor of metalloproteinases (TIMP) by human synovial fibroblasts is modulated by all-trans-retinoic acid. Biochim. Biophys. Acta, *1133:* 25–30, 1991.
21. Whitelock, J. M., O'Grady, R. L., and Gibbons, J. R. The identification, purification and characterization of an inhibitor of collagenase (20K) produced by neoplastic epithelial cells. Biochim. Biophys. Acta, *1073:* 107–113, 1991.
22. Korczak, B., Kerbal, R. S., and Dennis, J. Constitutive expression and secretion of proteases in non-metastatic SP1 mammary carcinoma cells and its metastatic sublines. Int. J. Cancer, *48:* 557–561, 1991.
23. Albini, A., Iwamoto, Y., Kleinman, H. K., Martin, G. R., Aaronson, S. A., Kozlowski, J. M., and McEwan, R. M. A rapid *in vitro* assay for quantitating the invasive potential of tumor cells. Cancer Res., *47:* 3239–3245, 1987.
24. Gleason, D. F. Classification of prostate carcinomas. Cancer Chemother. Rep., *50* (Part 1): 125–130, 1966.
25. Hammond, S. L., Ham, R., and Stamper, M. R. Serum-free growth of human mammary epithelial cells: rapid clonal growth in defined medium and extended serial passage with pituitary extract. Proc. Natl. Acad. Sci. USA, *81:* 5435–5439, 1984.
26. Bradford, M. A rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of protein-dye binding. Anal. Biochem., *72:* 248–254, 1976.
27. Sellers, A., Reynolds, J. J., and Meike, M. C. Neutral metalloproteinases from rabbit bone separation in latent forms of distinct enzymes that when activated degrade collagen, gelatin and proteoglycans. Biochem. J., *171:* 493–496, 1978.
28. Dean, D. D., and Woessner, J. F., Jr. A sensitive, specific assay for tissue collagenases using telopeptide-free ^{3}H-acetylated collagen. Anal. Biochem., *148:* 174–181, 1985.
29. Gunja-Smith, Z. Nagase, H., and Woessner, F. J., Jr. Purification of the neutral proteoglycan-degrading metalloproteinase from human articular cartilage tissue and its identification as stromelysin matrix metalloproteinase-3. Biochem. J., *258:* 115–119, 1989.
30. Zu, C., and Woessner, J. F., Jr. A tissue inhibitor of metalloproteinases and α-macroglobulins in the ovulating rat ovary: possible regulators of collagen matrix breakdown. Biol. Reprod., *45:* 334–342, 1991.
31. Herron, G. S., Werb, Z., Dwyer, K., and Banda, M. J. Secretion of metalloproteinases by stimulated capillary endothelial cells. II. Expression of collagenase and stromelysin activities is regulated by endogenous inhibitors. J. Biol. Chem., *261:* 2814–2818, 1986.
32. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (Lond.), *227:* 680–685, 1970.
33. Heussen C., and Dowdle, E. B. Electrophoretic analysis of plasminogen activators in polyacrylamide gels containing sodium dodecyl sulfate and copolymerized substrates. Anal. Biochem., *102:* 196–202, 1980.

34. Talhouk, R. S., Chin, J. R., Unemori, E. N., Werb, Z., and Bissell, M. J. Proteinases of the mammary gland: developmental regulation *in vivo* and vectorial secretion in culture. Development (Camb.), *112:* 439–449, 1991.
35. Nagase, H., and Woessner, J. F., Jr. Role of endogenous proteinases in the degradation of cartilage matrix. *In:* J. F. Woessner, Jr., and D. S. Howell (eds.), Joint Cartilage Degradation—Basic and Clinical Aspects, pp. 159–185. New York: Marcel Decker Inc., 1993.
36. Lefebvre, V., and Vaes, G. The enzymatic evaluation of procollagenase and collagenase inhibitors in crude biological media. Biochim. Biophys. Acta, *992:* 355–361, 1989.
37. Kleiner, D. E., Jr., Unsworth, E. J., Krutzsch, H. C., and Stetler-Stevenson, W. G. Higher-order complex formation between the 72-kilodalton type IV collagenase and tissue inhibitor of metalloproteinase-2. Biochemistry, *31:* 1665–1672, 1992.
38. Jones, E. G., and Harper, M. E. Studies on the proliferation, Secretory activities and epidermal growth factor receptor expression in benign prostatic hyperplasia explant cultures. Prostate, *20:* 133–149, 1992.
39. Sherwood, E. R., Theyer, G., Steiner, G., Berg, L. A., Kozlowski, J. M., and Lee, C. Differential expression of specific cytokeratin polypeptides in the basal and luminal epithelia of the human prostate. Prostate, *18:* 303–314, 1991.
40. Pajouh, M. S., Nagle, R. B., Breathnach, R., Finch, J. S., Brawer, M. K., and Bowden, G. T. Expression of metalloproteinase genes in human prostate cancer. J. Cancer Res. Clin. Oncol., *117:* 144–150, 1991.
41. Stearns, M. E., and Wang, M. Type IV collagenase (M_r 72,000) expression in human prostate: benign and malignant tissue. Cancer Res., *53:* 878–883, 1993.
42. Clark, I. M., Wright, J. K., Cawston, T. E., and Hazleman, B. L. Polyclonal antibodies against human fibroblast collagenase and the design of an enzyme-linked immunosorbent assay to measure TIMP-collagenase complex. Matrix, *12:* 108–115, 1992.
43. Barsky, S., Siegal, G., Jannotta, F., and Liotta, L. Loss of basement membrane components by invasive tumors but not by their benign counterparts. Lab. Invest., *49:* 140, 1983.
44. Sinha, A., Gleason, D. F., DeLeon, D. J., Wilson, M. J., Limas, C. Reddy, P. K., and Furcht, L. T. Localization of type IV collagen in the basement membrane of human prostate and lymph nodes by immunoperoxidase and immunoalkaline phosphatase. Prostate, *18:* 93–104, 1991.
45. Levy, A. T., Cioce, V., Sobel, M. E., Gabrisa, S., Grigioni, W. F., Liotta, L., and Stetler-Stevenson, W. G. Increased expression of the M_r 72,000 type IV collagenase in human colonic adenocarcinoma. Cancer Res., *51:* 439–444, 1991.
46. Stearns, M. E., and Wang, M. Regulation of kinesin expression and type IV collagenase secretion in invasive human prostate PC3 tumor sublines. Cancer Res., *51:* 5866–5875, 1991.
47. Hoosein, N. M., Boyd, D. D., Hollas, W. J., Mazar, A., Henkin, J., and Chung, L. W. K. Involvement of urokinase and its receptor in the invasiveness of human prostatic' carcinoma cell lines. Cancer Commun., *3:* 255–264, 1991.
48. Liotta, L. A., Steeg, P. S., and Stetler-Stevenson, W. G. Cancer metastasis and angiogenesis: an imbalance of positive and negative regulation. Cell, *64:* 327–336, 1991.
49. Alvarez, O. A., Carmichael, D. F., and DeClerck, Y. A. Inhibition of collagenolytic activity and metastasis of tumor cells by a recombinant human tissue inhibitor of metalloproteinases. J. Natl. Cancer Inst., *82:* 589–595, 1990.
50. Khokha, R., Zimmer, M. J., Graham, C. H., Lala, P. K., and Waterhouse, P. Suppression of invasion by inducible expression of tissue inhibitor of metalloproteinase-1 (TIMP-1) in B-16 F10 melanoma cells. J. Natl. Cancer Inst., *84:* 1017–1022, 1992.
51. Melchiori, A., Albini, A., Ray, J. M., and Stetler-Stevenson, W. O. Inhibition of tumor cell invasion by a highly conserved peptide sequence from the matrix metalloproteinase enzyme prosegment. Cancer Res., *52:* 2353–2356, 1992.