American Journal of Pathology, Vol. 144, No. 3, March 1994

Increased Expression of the 72-kd Type IV Collagenase in Prostatic Adenocarcinoma

Demonstration by Immunohistochemistry and in Situ *Hybridization*

A. H. Boag and I. D. Young

From the Department of Pathology, Kingston General Hospital and Queen's University, Kingston, Ontario, Canada

The expression of the 72-kd type IV collagenase has been implicated as an important factor in determining the invasive potential of malignant tumors. Using immunohistochemistry and nonisotopic* in situ *hybridization, type IV collagenase expression was assessed in benign and malignant prostatic tissue obtained from 117 surgical and autopsy specimens. Diffuse strong staining for type IV collagenase mRNA and protein was identified in the malignant cells of more than 85% of prostatic adenocarcinomas and the dysplastic cells of high grade prostatic intraepithelial neoplasia. Benign hyperplastic epithelium showed moderate expression in basal cells and mild expression in secretory cells. The qualitative patterns of type IV collagenase expression in prostatic epithelium at the protein and mRNA levels in individual cases were identical. There was no correlation between the level of type IV collagenase expression and either tumor grade or stage. In 10% of adenocarcinomas, focal mild to moderate stromal cell immunoreactivity was present but mRNA was not detectable in the stromal compartment in any case. The enhanced expression of type IV collagenase in dysplastic epithelium and prostatic adenocarcinoma suggests it contributes to the development of the invasive phenotype. The vast majority of the enzyme present in these tumors is synthesized by malignant cells and its production by stromal cells is negligible. (Am J Pathol 1994, 144:585–591)

Degradation of the extracellular matrix is required for tumor invasion. The matrix metalloproteinases (MMPs) comprise a group of related enzymes that appear to have an important role in this process.[1,2] The 72-kd type IV collagenase (cIVase) is an MMP that cleaves type IV collagen, the main structural component of the basement membrane. Because the basement membrane is the first matrix compartment to be breached during tissue invasion by carcinomas, increased cIVase activity may represent a critical early event in the development of the invasive phenotype. The cIVase activity of tumor cells has been shown to correlate with their ability to cross basement membranes *in vitro* and produce metastases in animal models.[3] In several human tumors including breast,[4] colonic,[5] ovarian,[6] and hepatocellular carcinomas,[7] cIVase expression has positively correlated with tumor invasiveness.

The relative contribution of neoplastic, stromal, and inflammatory cells to cIVase activity in human tumors remains unclear. By immunohistochemistry, cIVase expression has been shown to be largely restricted to malignant cells in breast, colonic, and hepatocellular carcinomas.[4,5,7] However, *in situ* hybridization (ISH) studies of skin, lung, and colorectal carcinomas have localized cIVase transcripts predominantly in stromal cells,[8–10] suggesting the mesenchymal compartment is the primary site of cIVase synthesis in these tumors.

The factors that determine the heterogeneous metastatic potential of prostatic adenocarcinoma are poorly understood, although it has been shown that progressive basement membrane loss occurs with increasing Gleason grade,[11] which in turn correlates with invasive potential. cIVase has previously been shown to be present in prostatic carcinomas and absent in normal prostates by Northern analysis.[12] We

Supported by the Clare Nelson Bequest Research Fund, Kingston General Hospital.

Accepted for publication November 22, 1993.

Address reprint requests to Dr. A. H. Boag, Department of Pathology, Queen's University, Kingston, Ontario, Canada.

have recently demonstrated enhanced expression of clVase in prostatic adenocarcinoma relative to benign prostatic epithelium using immunohistochemistry.[13] In this study we have used a combination of nonisotopic ISH and immunohistochemistry to extend the analysis of clVase in prostatic adenocarcinomas of varying grades and stages as well as normal and hyperplastic prostates. The results demonstrate that clVase expression is up-regulated at the mRNA and protein level in dysplastic and malignant epithelial cells but is not synthesized in significant amounts by tumor stroma.

Materials and Methods

Tissue

A retrospective immunohistochemical analysis of clVase expression was performed on 95 archival cases (including the 50 cases previously reported[13]) comprising 10 normal prostates (from autopsies on men under the age of 45 years), 29 cases of prostatic hyperplasia, and 56 cases of prostatic adenocarcinoma of varying grades and stages. All cases had been fixed in 10% neutral-buffered formalin and tissue sections were cut onto poly-L-lysine-coated slides.

In addition, immunohistochemistry and mRNA ISH were conducted on benign and malignant prostatic tissue derived from 22 prospectively gathered fresh radical prostatectomy specimens. Tissue blocks were fixed in 4% paraformaldehyde in phosphate-buffered saline, pH 7.0, for 4 hours at room temperature, processed using standard techniques and embedded in paraffin. Sections were cut onto 3-aminopropyltriethoxysilane (Aldrich Chemical, Milwaukee, WI)-coated slides.

Antibodies

Two polyclonal affinity-purified antitype IV collagenase antibodies were a generous gift of Dr. W. G. Stetler-Stevenson of the National Cancer Institute, NIH. Antibodies KLH 31 and KLH 48 were raised against synthetic peptides corresponding to the 17 NH_2-terminal amino acids of type IV procollagenase and the putative metal binding domain of clVase, respectively.[4]

Immunohistochemistry

Tissue sections were deparaffinized then rehydrated and blocked with 0.45% H_2O_2 in methanol for 30 minutes followed by normal goat serum (Dako Labs, Carpenteria, CA) diluted 1/20 for 20 minutes. The primary antibodies KLH 31 and KLH 48, diluted to 1 and 15 mg/ml, respectively, were applied and incubated at 4 C overnight. A 15-minute preincubation at 37 C was performed for KLH 48. A secondary biotinylated antibody (goat anti-rabbit, Zymed, San Francisco, CA), diluted 1/200, and peroxidase-conjugated streptavidin (Dakopatts, Glastrup, Denmark), diluted 1/300, were applied sequentially for 30 minutes, followed by 0.33 mg/ml 3–3′ diaminobenzidine tetrahydrochloride (Sigma, St. Louis, MO) in 50 nM Tris, pH 7.6, containing 0.045% H_2O_2 for 8 minutes. The sections were counterstained lightly with hematoxylin. Phosphate-buffered saline was used to dilute the antibodies and to rinse sections between steps. The primary antibodies were replaced by normal rabbit serum for negative control slides. Immunoreactivity was assessed using routine light microscopy and intensity graded from 0 to 4+.

Synthesis of Digoxigenin-Labeled cRNA Probes

Sense and antisense cRNA probes were transcribed from a polymerase chain reaction (PCR)-amplified segment of the clVase gene using a previously described technique.[14] Briefly, a 175 base sequence of the gene including nucleotides 2250–2425[15,16] was amplified from crude genomic DNA using 20-mer oligonucleotide sense and antisense primers. This sequence resides within a larger cRNA probe that was used previously for ISH.[9] The SP6 and T7 RNA polymerase promoters were incorporated into the amplified DNA by including these sequences in the 5′ termini of the sense and antisense primers, respectively. The PCR reaction mixture included: 40 µl of diethylpyrocarbonate (DEPC)-treated water, 10 µl of PCR buffer (650 mM Tris, pH 8.0, 20 mM $MgCl_2$, 160 mM ammonium sulfate, and 0.7% β-mercaptoethanol), 16 µl of 800 mM deoxyribonucleotide triphosphate mixture, 1 µl (20 pmol) of each primer, 30 µl of crude DNA extract, and 0.5 µl (2.5 U) Taq polymerase (Perkin-Elmer Cetus, Norwalk, CT). The DNA extract was obtained by boiling human blood in an equal volume of water for 10 minutes. After centrifugation, the supernatant was used as the template for the PCR. The reaction was performed for 30 cycles using a DNA thermal cycler (Perkin-Elmer Cetus) using the following profile: 94 C for 2 minutes, 50 C for 2 minutes, and 72 C for 1 minute lengthened by 5 seconds in each successive cycle. Analysis of the PCR product by polyacrylamide gel electrophoresis

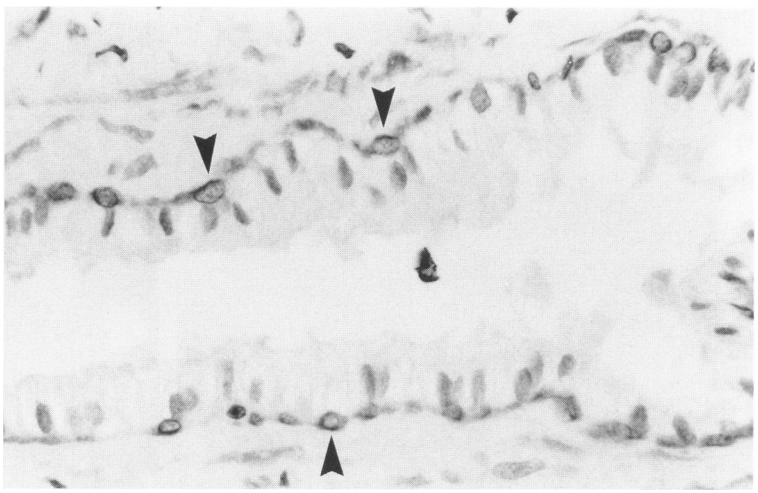


Figure 1. *Normal prostate gland showing moderate immunoreactivity for cIVase in basal cells (arrowheads) and minimal immunoreactivity in secretory epithelium (×400, hematoxylin counterstain).*

demonstrated a single, discrete DNA band of predicted size (data not shown).

Digoxigenin-labeled cRNA probes were transcribed from the amplified template by incubation of the following reaction mixture at 37 C for 2 hours: 1 µl PCR product, 2 µl transcription buffer (400 mM Tris-HCl, pH 8.0, 60 mM $MgCl_2$, 20 mM spermidine, 100 mM NaCl), 2 µl ribonucleotide triphosphate mixture (10 mM ATP, 10 mM GTP, 10 mM CTP, 3.5 mM digoxigenin-11-UTP, Boehringer Mannheim, Mannheim, Germany, 6.5 mM UTP), 40 U of SP6 or T7 RNA polymerase (Bio/Can Scientific, Inc., Mississauga, Ontario, Canada), and DEPC-treated water to a final volume of 20 µl. DNA was digested with 20 U of RNAse-free DNAse (Pharmacia, Uppsala, Sweden) for 20 minutes at 37 C and the DNAse inactivated by

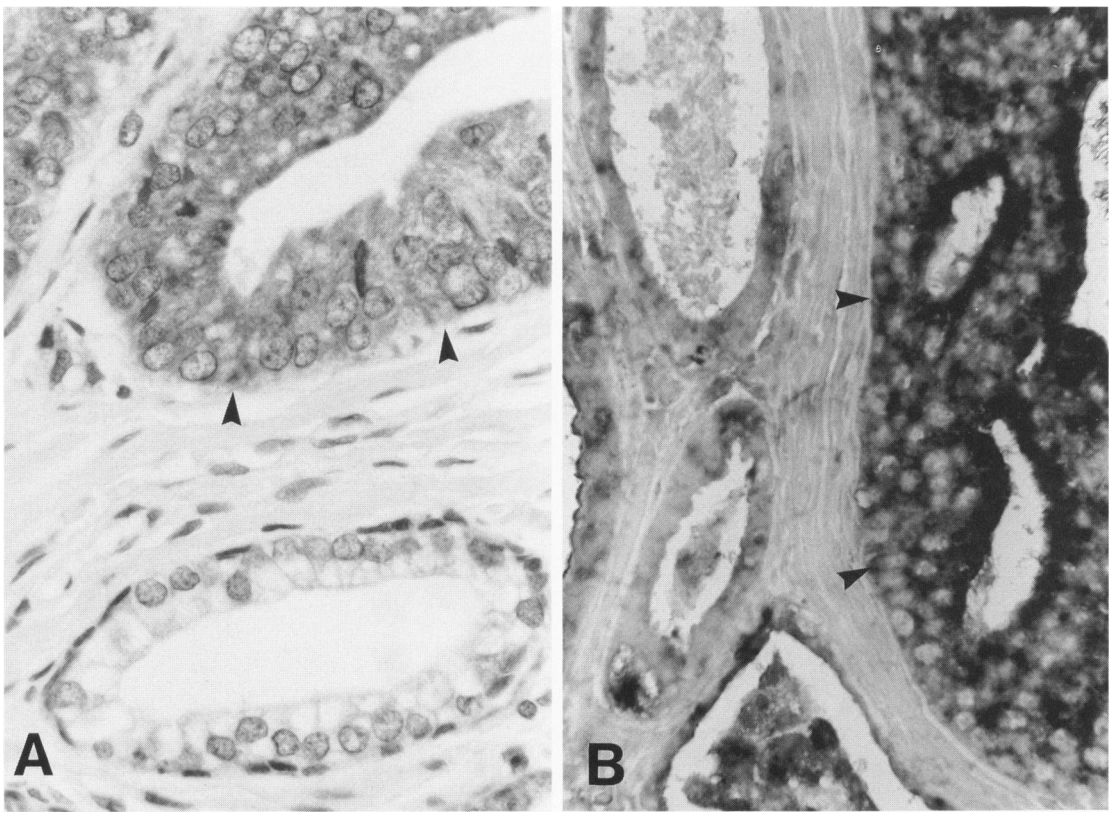
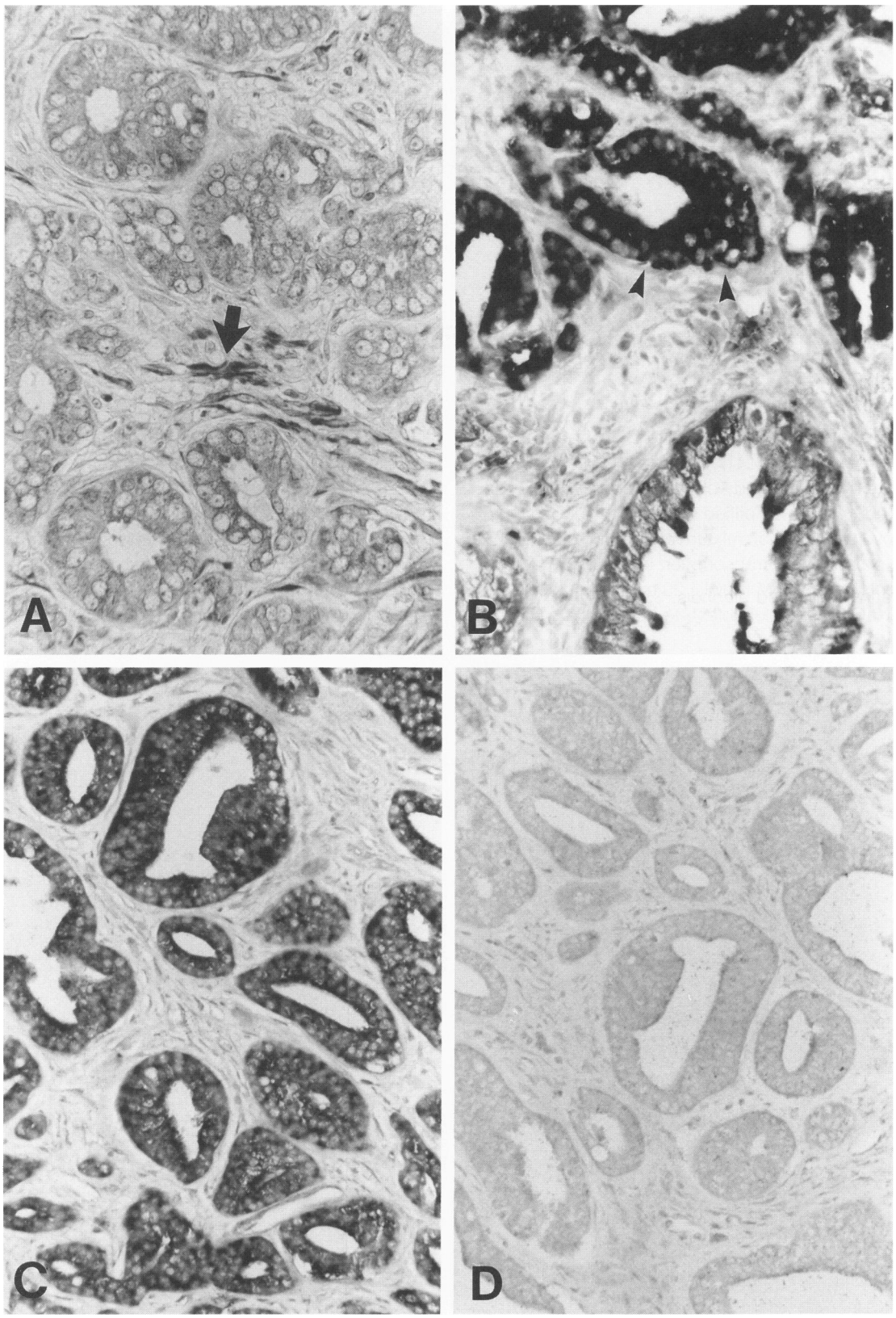


Figure 2. *High grade prostatic intraepithelial neoplasia (arrowheads) shows high levels of cIVase expression by both immunohistochemistry (A) and ISH (B). Adjacent stroma and benign glands show minimal expression (A, ×250, hematoxylin counterstain; B, ×200, methyl green counterstain).*


A
B
C
D

heating to 65 C for 5 minutes. The probes were precipitated at −20°C overnight after addition of 1/8 volume of 4 M LiCl and three volumes of cold absolute ethanol. The precipitate was washed three times in 75% ethanol, dissolved in DEPC-treated water, and the probe concentration determined by measuring optical density at 260 nM. To verify the identity of antisense probe. Northern blots of the obtained transcripts were hybridized under high stringency conditions with a [δ-^{32}] PATP end-labeled 18-mer oligonucleotide complementary to an internal sequence of the antisense transcript. This internal oligonucleotide showed specific hybridization to the antisense transcript (data not shown).

ISH

Tissue sections were deparaffinized, rehydrated, washed in 0.2 M HCl for 10 minutes, and rinsed in nanopure water. They were then washed with 0.25% acetic anhydride in buffer 1 (150 mM NaCl, 100 mM Tris-HCl, pH 7.5) for 10 minutes then rinsed in buffer 1 and washed in 0.2% glycine in buffer 1. After dehydration in graded alcohols, 30 µl of hybridization buffer (50 mM Tris, pH 7.6, 1 mM ethylenediamine tetraacetic acid, 0.02% Ficoll, 0.02% polyvinylpyrolidone, 500 µg/ml yeast tRNA [Boehringer Mannheim], 10 mM dithiothreitol, 0.3 M NaCl, 10% dextran sulfate, and 50% formamide) containing 500 µg/ml boiled sheared salmon sperm DNA (Sigma) was applied per cm^2 of tissue section. The slides were placed in a metal pan that was floated in a 95 C water bath for 10 minutes. Excess prehybridization solution was drawn off the slides and 30 µl of hybridization buffer containing 20 µg/ml denatured probe was applied. The hybridization was performed at 50 C for 2 hours in a humidified box. In addition to using sense probes, negative controls included elimination of the antisense probe and pretreatment of sections with RNase A (Sigma) before hybridization.

After hybridization, sections were washed sequentially with 2× standard saline citrate (SSC) (1× SSC is 0.15 M NaCl, 0.015 M sodium citrate) for 5 minutes and 0.2× SSC for 30 minutes at room temperature. Sections were blocked with 1% casein (U.S. Biochemical Corp., Cleveland, OH) in buffer 1 for 30 minutes and then 100 µl of alkaline phosphatase-conjugated antidigoxigenin F(ab) fragments (Boehringer Mannheim) diluted 1:1000 in buffer 1 was applied for 2 hours. The slides were washed twice in buffer 1 for 15 minutes and rinsed in buffer 2 (100 nM NaCl, 20 nM $MgCl_2$, 100 nM Tris-HCl, pH 9.5). The sections were covered with 100 µl chromogen substrate solution (45 µl of 90 mM nitroblue tetrazolium salt in 70% [volume/volume] dimethylformamide and 35 µl of 120 mM 5-bromo-4-chloro-3-indolylphosphate toludinium salt in 100% dimethylformamide in 10 ml buffer 2) containing 0.5 mM levamisole and incubated overnight at room temperature. The sections were rinsed well with nanopure water, counterstained with 2% chloroform-extracted methyl green, and coverslipped with an aqueous mounting medium.

Results

Immunohistochemistry

Most (over 75%) basal epithelial cells in all cases of normal and hyperplastic prostatic tissue showed moderate (3+) cytoplasmic immunoreactivity for cIVase (Figure 1). Acinar and ductal secretory epithelial cells demonstrated immunostaining of lower intensity (1 to 2+), although these cells did show moderate immunoreactivity in some cases. Atrophic epithelium showed absent to focal slight (0 to 1+) immunoreactivity. Endothelium and transitional epithelium lining the urethra and distal prostatic ducts showed moderate immunostaining. The stroma in normal prostates and most cases of hyperplasia showed no immunoreactivity. However, in 10% of cases of hyperplasia, focal areas of moderate cytoplasmic immunoreactivity in stromal cells were identified, a finding that was more pronounced with antibody KLH 31 than KLH 48.

In 96% of the archival cases of prostatic adenocarcinoma and 85% of paraformaldehyde-fixed cases, tumor cells showed uniformly moderate to strong (3 to 4+) cytoplasmic immunoreactivity. Tissue fixation in the negative cases was suboptimal and may have contributed to the lack of staining. The overall staining intensity was less in paraformaldehyde-fixed than formalin-fixed tissue. High grade prostatic intraepithelial neoplasia (PIN) showed strong immunoreactivity similar to that seen in invasive cancers and significantly more intense than adjacent nondysplastic ductal epithelium (Figure 2A). There was no

Figure 3. **A:** *Prostatic adenocarcinoma demonstrates diffuse immunoreactivity for cIVase in malignant epithelial cells and focal immunoreactivity in stromal cells (arrow).* **B:** *ISH localizes very abundant cIVase mRNA in the epithelial compartment of prostatic adenocarcinoma (arrowheads) compared with minimal expression in adjacent benign epithelium.* **C:** *ISH in the case depicted in* (**A**) *shows cIVase mRNA is restricted to neoplastic epithelium with no stromal expression apparent.* **D:** *ISH with sense cRNA shows no staining* (**A**, ×*200, hematoxylin counterstain;* **B** *to* **D**, ×*200, methyl green counterstain*).

correlation between cIVase immunoreactivity and tumor grade or stage, although a trend to decreased expression was seen in some very poorly differentiated carcinomas. Tumor infiltrating periprostatic tissue, and foci of metastatic carcinoma in lymph nodes and soft tissue, all showed immunoreactivity of similar intensity to tumor within the prostate.

Prostatic stromal cells in malignant prostates were generally negative. However, in 10% of carcinomas there were focal areas of slight to moderate cytoplasmic immunostaining of stromal cells with KLH 31 but not KLH 48 (Figure 3A). Mesenchymal cells associated with foci of metastatic tumor showed no immunoreactivity.

ISH

There was a strong positive correlation between the ISH and immunohistochemistry results in both carcinomas and benign epithelium. High levels of cIVase mRNA, as indicated by dense cytoplasmic staining of malignant cells, were apparent in 85% of prostatic carcinomas. There was no hybridization in two highly anaplastic carcinomas and in two cases in which tissue fixation was suboptimal. Apart from a trend to decreased expression in very poorly differentiated tumors, no correlation of mRNA level with grade was found. Metastatic tumor in the single pelvic lymph node studied and foci of locally invasive tumor in the urinary bladder wall in a second case showed similar intensities of staining to the corresponding primary tumors within the prostate. There was no correlation between the ISH results and pathological stage. Foci of PIN also expressed abundant cIVase mRNA, whereas hyperplastic epithelium showed mild to moderate positivity and atrophic glands were generally negative (Figure 2B). The ISH signal intensity was clearly lower in benign prostatic epithelium when compared with prostatic adenocarcinoma.

ISH detected no cIVase mRNA in any stromal cells in either benign or malignant prostates, including those cases in which focal stromal cell immunoreactivity for cIVase was identified (Figure 3).

ISH performed using the sense probe as a negative control showed minimal background staining (Figure 3D). Treatment of tissue sections with RNase A before hybridization completely eliminated the signal (data not shown). The specificity of the ISH is also supported by the excellent correlation with the immunohistochemistry performed on the same tissue samples. The two anaplastic carcinomas in which ISH was negative showed weak staining by immunohistochemistry and two cases in which fixation appeared poor show minimal staining by both methods. Otherwise, all cases were positive by both techniques.

Discussion

cIVase is thought to play a role in the proteolysis of basement membrane by malignant cells that occurs early in the development of invasive tumors. The results of the current study demonstrate that cIVase expression is enhanced at both the protein and mRNA levels in prostatic adenocarcinoma, consistent with such a role for cIVase in this cancer. High grade PIN was also found to display markedly elevated levels of cIVase. This probable precursor of prostatic adenocarcinoma[17] is analogous to atypical epithelial hyperplasia and *in situ* carcinoma of the breast in which increased levels of cIVase have also been found.[4] These data indicate that overexpression of cIVase is an early event that precedes the invasion of carcinomas in general and prostatic adenocarcinomas in particular.

Similar levels of expression of cIVase were found in well differentiated tumors restricted to the prostate and poorly differentiated tumors with extensive local invasion and distant metastases. This contrasts with the findings of studies on hepatocellular carcinoma[7] and colonic carcinomas[5,9] in which increased cIVase expression correlated with higher tumor grades and stages. Other variables are presumably involved in determining the full invasive phenotype of prostatic adenocarcinomas. A tipping of the balance between cIVase activation and inhibition could in part explain the heterogeneous invasive potential in prostatic carcinomas that show similar levels of cIVase protein and mRNA.[18,19] Other possible factors could include differential expression of other matrix metalloproteases or molecules involved in cell adhesion and migration.

Further evidence that elevated cIVase levels alone do not confer invasive behavior is provided by the finding of moderate levels of cIVase expression in basal cells. This probably reflects a greater role in normal ongoing basement membrane metabolism for these cells compared with the secretory cells that generally showed lower levels of cIVase expression. An analogous observation has been made in normal breast tissue in which the myoepithelial cells show higher levels of cIVase expression when compared with the secretory epithelium.[4]

The ISH data from this study suggest that cIVase production in prostatic adenocarcinoma occurs ex-

clusively in the neoplastic cells. It is possible that cIVase transcripts are present in very low abundance in prostatic stromal cells and that our ISH technique was inadequately sensitive to detect them. However, a recent study using Northern blot analysis has also suggested that cIVase mRNA is largely restricted to the neoplastic cells in prostatic adenocarcinoma.[20] In previous studies of skin, lung, and colorectal carcinomas,[8–10] ISH localized cIVase mRNA principally in tumor stromal cells. Immunoreactive cIVase was identified primarily in neoplastic cells, suggesting that cIVase synthesized and secreted by tumor stroma was secondarily taken up by malignant cells. In prostatic adenocarcinoma, however, malignant prostatic epithelial cells express both abundant cIVase mRNA and protein, demonstrating they are producing the enzyme. Stromal cells in prostatic tumors are not a significant source of cIVase.

In summary, prostatic adenocarcinomas showed increased expression of cIVase, which is produced almost exclusively by neoplastic cells. Similar levels of cIVase are present in high grade PIN and invasive carcinomas of varying grades and stages, indicating that although cIVase may play a role in prostate cancer invasion, other factors must be involved in determining the full invasive potential of this tumor.

References

1. Woessner JF: Matrix metalloproteinases and their inhibitors in connective tissue remodeling. FASEB J 1991, 5:2145–2154
2. Matrisian LM: The matrix-degrading metalloproteinases. Bioessays 1992, 14:455–463
3. Stetler-Stevenson WG: Type IV collagenase in tumor invasion and metastases. Cancer Metastasis Rev 1990, 9:289–303
4. Monteagudo C, Merino MJ, San-Juan J, Liotta LA, Stetler-Stevenson WG: Immunohistochemical distribution of type IV collagenase in normal, benign, and malignant breast tissue. Am J Pathol 1991, 136:585–592
5. Levy AT, Cioce V, Sobel ME, Garbisa S, Grigioni WF, Liotta LA, Stetlerstevenson WG: Increased expression of the Mr 72,000 type-IV collagenase in human colonic adenocarcinoma. Cancer Res 1991, 51:439–444
6. Campo E, Merino MJ, Tavassoli FA, Charonis AS, Stetler-Stevenson WG, Liotta LA: Evaluation of basement membrane components and the 72-kDa type-IV collagenase in serous tumors of the ovary. Am J Surg Pathol 1992, 16:500–507
7. Grigioni WF, Garbisa S, D'Errico A, Baccarini P, Stetler-Stevenson WG, Liotta LA, Mancini AM: Evaluation of hepatocellular carcinoma aggressiveness by a panel of extracellular matrix antigens. Am J Pathol 1991, 138:647–654
8. Pyke C, Ralfkiaer E, Huhtala P, Hurskainen T, Dano K, Tryggvason K: Localization of messenger RNA for Mr 72,000 and 92,000 type-IV collagenases in human skin cancers by insitu hybridization. Cancer Res 1992, 52:1336–1341
9. Poulson R, Pignatelli M, Stetler-Stevenson WG, Liotta LA, Wright PA, Jeffery RE, Longcroft JM, Rogers L, Stamp GWH: Stromal expression of 72 kda type-IV collagenase (MMP-2) and TIMP-2 messenger RNAs in colorectal neoplasia. Am J Pathol 1992, 141:389–396
10. Soini Y, Paakko P, Autio-Harmainen H: Genes of laminin B1 chain, a1 (IV) chain of type iv collagen, and 72-kd type IV collagenase are mainly expressed by the stromal cells of lung carcinomas. Am J Pathol 1993, 142:1622–1630
11. Fuchs ME, Brawer MK, Rennels MA, Nagle RB: The relationship of basement membrane to histologic grade of human prostatic carcinoma. Mod Pathol 1989, 2:105–109
12. Pajouh MS, Nagle RB, Breathnach R, Finch JS, Brawer MK, Bowden GT: Expression of metalloproteinase genes in human prostate cancer. J Cancer Res Clin Oncol 1991, 117:144–150
13. Boag AH, Young ID: Immunohistochemical analysis of type IV collagenase expression in prostatic hyperplasia and adenocarcinoma. Mod Pathol 1993, 6:65–68
14. Young ID, Ailles L, Deugau K, Kisilevsky R: Transcription of cRNA for insitu hybridization from polymerase chain reaction-amplified DNA. Lab Invest 1991, 64:709–712
15. Collier IE, Wilhelm SM, Eisen AZ, Marmer BL, Grant GA, Seltzer JL, Kronberger A, He C, Bauer EA, Goldberg GL: H-*ras* oncogene-transformed human bronchial epithelial cells (TBE-1) secrete a single metalloprotease capable of degrading basement membrane collagen. J Biol Chem 1988, 263:6579–6587
16. Collier IE, Bruns GAP, Goldberg GI, Gerhard DS: On the structure and chromosome location of the 72-kDa and 92-kDa human type-IV collagenase genes. Genomics 1991, 9:429–434
17. McNeal JE, Bostwick DG: Intraductal dysplasia: a premalignant lesion of the prostate. Hum Pathol 1986, 17: 64–71
18. Liotta LA, Steeg PS, Stetler-Stevenson WG: Cancer metastases and angiogenesis: an imbalance of positive and negative regulation. Cell 1991, 64:327–336
19. Howard EW, Bullen EC, Banda MJ: Preferential inhibition of 72-kDa and 92-kDa gelatinases by tissue inhibitor of metalloproteinases-2. J Biol Chem 1991, 266:13070–13075
20. Stearns ME, Wang M: Type IV collagenase (mr 72,000) expression in human prostate benign and malignant tissue. Cancer Res 1993, 53:878–883