ADAM 12 Cleaves Extracellular Matrix Proteins and Correlates with Cancer Status and Stage*

Received for publication, August 19, 2004
Published, JBC Papers in Press, September 20, 2004, DOI 10.1074/jbc.M409565200

Roopali Roy‡‡, Ulla M. Wewer¶¶, David Zurakowski§§, Susan E. Pories‡‡‡, and Marsha A. Moses§§§

From the ‡Program in Vascular Biology and Department of Surgery and the §§Departments of Orthopedic Surgery and Biostatistics, Children’s Hospital, Boston, Massachusetts 02115, ¶¶Beth Israel Deaconess Medical Center and Mount Auburn Hospital, Boston, Massachusetts 02138, §§§Harvard Medical School, Boston, Massachusetts 02115, and §§ Institute of Molecular Pathology, University of Copenhagen, Copenhagen, Denmark

ADAM 12 is a member of a family of disintegrin-containing metalloproteases that have been implicated in a variety of diseases including Alzheimer’s disease, arthritis, and cancer. We purified ADAM 12 from the urine of breast cancer patients via Q-Sepharose anion exchange and gelatin-Sepharose affinity chromatography followed by protein identification by matrix-assisted laser desorption/ionization-time of flight mass spectrometry. Four peptides were identified that spanned the amino acid sequence of ADAM 12. Immunoblot analysis using ADAM 12-specific antibodies detected an ~68-kDa band identified as the mature form of ADAM 12. To characterize catalytic properties of ADAM 12, full-length ADAM 12-S was expressed in COS-7 cells and purified. Substrate specificity studies demonstrated that ADAM 12-S degrades gelatin, type IV collagen, and fibronectin but not type I collagen or casein. Gelatinase activity of ADAM 12 was completely abrogated by zinc chelators 1,10-phenanthroline and EDTA and was partially inhibited by the hydroxamate inhibitor Marimastat. Endogenous matrix metalloprotease inhibitor TIMP-3 inhibited activity. To validate our initial identification of this enzyme in human urine, 117 urine samples from breast cancer patients and controls were analyzed by immunoblot. The majority of samples from cancer patients were positive for ADAM 12 (67 of 71, sensitivity 0.94) compared with urine from controls in which ADAM 12 was detected with significantly lower frequency. Densitometric analyses of immunoblots demonstrated that ADAM 12 protein levels were higher in urine from breast cancer patients than in control urine. In addition, median levels of ADAM 12 in urine significantly increased with disease progression. These data demonstrate for the first time that ADAM 12 is a gelatinase, that it can be detected in breast cancer patient urine, and that increased urinary levels of this protein correlate with breast cancer progression. They further support the possibility that detection of urinary ADAM 12 may prove useful in the development of noninvasive diagnostic and prognostic tests for breast and perhaps other cancers.

ADAMs (a disintegrin and metalloprotease) are a family of integral membrane and secreted glycoproteins that are related to snake venom metalloproteases and matrix metalloproteases (MMPs). These proteases are multidomain proteins composed of a prodomain, metalloprotease domain, disintegrin domain, and a cysteine-rich region, and membrane-anchored ADAMs also contain a transmembrane and cytoplasmic domain. ADAMs display diverse roles in cell surface remodeling, ectodomain shedding, regulation of growth factor availability, and in mediating cell-cell and cell-matrix interactions in both normal development and pathological states such as Alzheimer’s disease, arthritis, cancer, and cardiac hypertrophy (1–3). Human ADAM 12 is expressed as two alternatively spliced forms: a membrane-anchored long form (ADAM 12-L) and a shorter secreted form (ADAM 12-S) (4). ADAM 12-S is an active protease known to cleave IGFBP-3 and IGFBP-5 (5–7). Recently, ADAM 12 has been reported to play a role in cardiac hypertrophy, cleaving membrane-bound heparin-binding-EGF in response to G protein-coupled receptor stimulation of cardiomyocytes leading to shedding of heparin-binding EGF from the cell surface and transactivation of the EGF receptor (8). Increased expression of ADAM 12 mRNA and protein has been reported in breast, colon, and lung carcinoma tissue (9). In vitro, the cysteine-rich domain of ADAM 12 has been shown to support tumor cell adhesion mediated through syndecans and integrin cell surface receptors to generate signals for cell adhesion and spreading (10). ADAM 12 mRNA levels are increased almost 6-fold in hepatocellular carcinoma, and its expression correlates with tumor aggressiveness and progression (11). MMPs, a class of matrix-degrading enzymes, play an important role in tumor growth and metastasis and are involved in the remodeling of the tumor microenvironment (12, 13). Elevated levels of MMPs have been measured in body fluids such as serum and plasma of human patients and in tumor-bearing animals. More recently, we have reported that MMPs can also be detected in urine of patients with a variety of cancers including prostate, bladder, renal, and breast and serve as independent predictors of disease status (14). Our research has also identified an ~125-kDa gelatinase species in urine of cancer patients as a complex of MMP-9 with neutrophil gelatinase-

* This work was supported in part by the Jo Ann Webb Fund for Kidney Research, the Russo Family Charitable Foundation, the Advanced Medical Foundation, and GMP Companies, Inc. (to M. A. M.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Supported by the Danish Cancer Society and the Danish Medical Research Council.

‡‡ To whom correspondence should be addressed: Program in Vascular Biology, Karp Family Research Bldg., Floor 12, Children’s Hospital, Harvard Medical School, 300 Longwood Ave., Boston, MA 02115. Tel.: 617-919-2207; Fax: 617-730-0231; E-mail: marsha.moses@childrens.harvard.edu.

The abbreviations used are: ADAM, a disintegrin and metalloprotease; ADAM 12-L, membrane-anchored long form of ADAM 12; ADAM 12-S, secreted form of ADAM-12; ADH, atypical ductal hyperplasia; LCIS, lobular carcinoma in situ; DCIS, ductal carcinoma in situ; IBC, locally invasive breast cancer; ROC, receiver operating characteristic; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; CHAPS, 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid; EGF, epidermal growth factor; MMP, matrix metalloprotease; ECM, extracellular matrix; TIMP, tissue inhibitor of metalloproteinases; IGFBP, insulin-like growth factor-binding protein.
associated lipocalin and showed its presence in urine to be predictive of disease status (15). A number of studies have confirmed these original findings in a variety of human cancers; however, the presence of other proteases as disease markers in urine has not yet been thoroughly explored.

The present study is the result of a biomarker identification initiative in our group whose goal is to identify proteins present in urine of cancer patients and to determine whether their presence might be relevant to disease status. Using anion exchange and affinity chromatographic steps followed by MALDI-TOF mass spectrometry, we identified ADAM 12 in urine from breast cancer patients. This result was confirmed by Western blot analysis of urine using ADAM 12-specific antibodies. Comparative analysis of urine from breast cancer patients and healthy individuals showed not only that detection of ADAM 12 in urine is indicative of disease status but also that levels of this enzyme in urine correlate with stages of disease.

The physiological substrate of ADAM 12 has not yet been determined. Given the importance of extracellular matrix degradation in tumorigenesis and metastasis we tested the ability of this metalloprotease to degrade ECM components. We found that ADAM 12 can degrade various ECM proteins including gelatin, type IV collagen, and fibronectin, suggesting a potential role for this enzyme in ECM remodeling, a hallmark of neoplastic disease.

**MATERIALS AND METHODS**

**Identification of Urinary ADAM 12**—Initial identification of ADAM 12 was made using 50 ml of urine (total protein ~5 mg). Urine was concentrated by ultrafiltration using an Amicon membrane XM-50 (Millipore Corporation, Bedford, MA). The sample was diluted with 2 volumes of 50 mM Tris, pH 7.5 (buffer A) and applied to a Q-Sepharose column (Pharmacia LKB, Piscataway, NJ). The sample was diluted with 2 volumes of buffer A containing 0.05% CHAPS. After washing, a gradient of 0-400 mM NaCl in buffer A was applied. Fractions containing gelatinase activity were pooled, the NaCl concentration was adjusted to 200 mM, and the fractions were incubated with gelatin-Sepharose (Amersham Biosciences) for 16 h at 4 °C with constant shaking. After a wash step, elution was performed using a stepwise gradient of 5, 10, and 20% MeSO4, respectively, in buffer A containing 200 mM NaCl. Eluted fractions were concentrated and separated via SDS-PAGE under non-reducing conditions and stained using the coomassie brilliant blue silver stain kit (Natiographics, Atlanta, GA) according to the manufacturer’s instructions. Several distinct bands of ~52, 80, 120, and 140 kDa were detected. Protein bands were excised from the gel, subjected to tryptic digest, and analyzed by MALDI-TOF mass spectrometry (Perceptive STR, Applied Biosystems, Framingham, MA) to determine the molecular weights of the proteins and for peptide mapping. The digest was run with a 337-nm wavelength laser for desorption and the reflectron mode of analysis. Using the MSFit search program the peptide maps generated were searched against a FASTA data base of public domain proteins constructed of protein sequences in the non-redundant data base held by the NCBI and Swiss-Prot. Peptide matches identified by MSFit were filtered according to their MOWSE (molecular weight/gauge) score, percentage of masses matched, molecular weight, and number of observations of peptides and proteins. For conservative identification of proteins at least two peptides with good matches identified by MSFit were filtered according to their MOWSE (molecular weight/gauge) score, percentage of masses matched, molecular weight, and number of observations of peptides and proteins. For conservative identification of proteins at least two peptides with good scores that match a single protein are required.

**Expression and Purification of ADAM 12-S**—The full-length human ADAM 12-S gene was cloned into a plasmid (p1151), and transfection of COS-7 cells (ATCC, CRL 1651) was conducted as described previously (5). Expressed recombinant ADAM 12-S secreted into the conditioned medium was purified as described previously with minor modifications. Briefly, conditioned medium from transfected COS-7 cells was subjected to successive steps of SP-Sepharose and concanavalin A-Sepharose chromatography as described (6). ADAM 12-S containing fractions from the concanavalin A-Sepharose column were pooled and after buffer exchange applied to a Vivaspin H Mini Q column (VivaScience, Carlsbad, CA) pre-equilibrated with buffer A containing 0.01% CHAPS. Pure ADAM 12-S was detected in the flow-through fraction as determined by SDS-PAGE and immunoblot analysis.

**Substrate Specificity Studies**—Gelatin-, type IV collagen-, fibronectin-, and casein-degrading activities of ADAM 12-S was measured as described previously (14). ADAM 12-S (1 μM) was mixed with non-reducing sample buffer and separated on a 10% polyacrylamide gel containing either 0.1% gelatin, 0.1% type IV collagen, or 0.02% fibronectin and a 12% polyacrylamide gel containing 0.1% casein. After electrophoresis, the gel was washed with 2.5% Triton X-100 for 30 min. Substrate digestion was conducted by incubating the gel in 50 mM Tris, pH 7.6, containing 5 mM CaCl2, 1 mM ZnCl2, and 0.02% NaN3 at 37 °C for 18 h. Gels were stained with 0.1% Coomassie, and bands of enzyme activity were detected as zones of clearance on a background of uniform blue staining. To determine whether ADAM 12 possessed type I collagen-degrading activity, pure enzyme (1 μM) was analyzed using a radiometric collagenase assay as described previously (16). For inhibition assays, ADAM 12 (160 nM) was incubated with 30 μg of gelatin in assay buffer (50 mM Tris, pH 7.5) in the presence or absence of various inhibitors: 5 mM 1,10-phenanthroline, 5 mM EDTA, 1 mM Marimastat, or two different concentrations (100 and 500 nM) of either TIMP-1, TIMP-2, TIMP-3, or TIMP-4 in a final volume of 30 μl at 37 °C for 18 h. Reactions were stopped with the addition of non-reducing sample buffer and the samples were resolved by SDS-PAGE analysis followed by Coomassie staining.

**Urine Sample Collection and Processing**—Urine collection was performed as described previously (14). Samples were collected in sterile containers and immediately frozen at ~20 °C. Urine was collected according to the institutional bioethical guidelines pertaining to discarded clinical material. Prior to analysis, urine was tested for the presence of blood and leukocytes using Multistix 9 urinalysis strips (Bayer, Tarrytown, NY) and samples containing blood or cells were excluded. Creatinine concentration of urine was determined using a commercial kit (Sigma) according to the manufacturer’s protocol. Protein concentration of urine was determined by the Bradford method using bovine serum albumin as the standard.

**Patient Population**—117 urine samples were analyzed for the presence of ADAM 12. The control group consisted of 46 women (median age 50 years) with no discernable disease. The breast cancer group included 71 patients diagnosed at various stages of breast cancer including atypical ductal hyperplasia (ADH), lobular carcinoma in situ (LCIS), ductal carcinoma in situ (DCIS), locally invasive breast cancer (IBC), and metastatic disease (see Fig. 3B).

**Western Blot Analysis**—Equal amounts of proteins (20 μg) were separated by SDSPAGE under reducing conditions. Resolved proteins were transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, NH) and treated as described previously (15). Labeled proteins were visualized with enhanced chemiluminescence (Pierce). Polyclonal antibodies against human ADAM 12, rb122 (4) and S18 (sc-16526, Santa Cruz Biotechnology), were used at 1 μg/ml and 2 μg/ml concentration, respectively. Band intensities were analyzed with UN-SCAN-IT (Silk Scientific, Orem, UT) software digitizer and ImageJ (U.S. National Institutes of Health).

**Statistical Analysis**—Sensitivity, specificity, false-positive rate, false-negative rate, and likelihood ratio were calculated using standard formulas. The presence of ADAM 12 was compared between cancer groups (ADH/LCIS, DCIS, IBC, and metastatic disease) and controls using logistic regression analysis. For each group, the presence of ADAM 12 was expressed as a percentage, and 95% confidence intervals were calculated based on the normal approximation. The nonlinear relationship between ADAM 12 level and breast cancer was modeled using logistic regression analysis with probability determined by maximum likelihood estimation for selected intervals of ADAM 12 (17). Median levels of ADAM 12 were compared between controls and cancer patients using the Kruskal-Wallis test with pairwise comparisons based on Mann-Whitney U tests, whereas mean levels were compared by analysis of variance. To evaluate the diagnostic accuracy of ADAM 12, receiver operating characteristic (ROC) curve analysis was utilized to plot all pairs of true positives and false positives for varying levels of ADAM 12. ROC curves were constructed for differentiating between all breast cancer patients and controls and separately for each cancer group. ROC curves that go up steeply from (0, 0) and reach near (1, 1) indicate a test that is almost perfect in discriminating cancer patients from controls, whereas curves close to the 45° diagonal line have low discriminatory power. Area under the curve with 95% confidence intervals were determined using the Hanley-McNeil algorithm as an index of the performance of ADAM 12 as a biomarker of disease. In addition, the area under the curve equals the probability that a randomly chosen pair of cancer patients and controls will be correctly classified. Actual numbers of cancer patients and controls are depicted as histograms with the theoretical probability curve superimposed to illustrate the increasing likelihood of breast cancer for higher levels of ADAM 12. To account for multiple comparisons among the five independent groups (four breast cancer groups according to stage of disease and the normal control group), a conservative two-tailed criterion of $p < 0.05 (0.05 divided by 5)$ was regarded as statistically significant. All statistical analysis was
performed using the SAS statistical package (version 6.12, SAS Institute, Cary, NC). A power analysis indicated that a minimum of 13 patients in each of the breast cancer stages would provide 80% power for detecting differences in ADAM 12 level (version 5.0, nQuery Advisor, Statistical Solutions, Boston, MA).

RESULTS

Isolation and Identification of ADAM 12—Urine from breast cancer patients was subjected to stepwise chromatography and in an effort to isolate proteases an affinity chromatography step was performed using gelatin-Sepharose. The fact that ADAM 12 bound gelatin-Sepharose beads supported our data (see below) that this enzyme has gelatin-degrading capability. The fraction collected when 20% Me₂SO was applied to the gelatin-Sepharose column contained several distinct (~52-, 80-, 120-, and 140-kDa protein bands (Fig. 1A, lane 2). MALDI-TOF analysis of the ~120-kDa protein band (Fig. 1A, lane 2), identified it as a disintegrin and metalloprotease domain 12 (ADAM 12) (accession NP_003465). Four distinct peptides spanning the amino acid sequence of human ADAM 12 were identified (Fig. 2A). When assayed by substrate zymography, ADAM 12 could proteolytically degrade gelatin. The purified human ADAM 12-S sample consisted of an ~68-kDa mature form of the enzyme that displayed a gelatinase activity band (Fig. 2B, lane 2). ADAM 12 also degraded fibronectin (Fig. 2B, lane 3) and type IV collagen (Fig. 2B, lane 4) but did not degrade casein (Fig. 2B, lane 5) when assayed by substrate zymography or type I collagen when tested in the radiometric collagenase assay (data not shown). The gelatin-degrading activity of ADAM 12 was used to evaluate the effect of different classes of protease inhibitors on this enzyme. Pure ADAM 12-S was incubated with gelatin (~140-kDa protein band, Fig. 2C, lane 2) at 37 °C for either 2 or 18 h in the absence (Fig. 2C, lanes 3 and 4) or presence of various inhibitors. Disappearance of the ~140-kDa band indicated that ADAM 12 cleaved gelatin in a time-dependent manner (Fig. 2C, lanes 3 and 4). The classical chelators of zinc metalloproteases, EDTA and 1,10-phenanthroline, inhibited gelatin degradation by ADAM 12, whereas the addition of 1 mM Marimastat resulted in partial inhibition of enzyme activity (Fig. 2C, lanes 5–7). The endogenous inhibitors TIMP-1, TIMP-2, and TIMP-4 did not inhibit the gelatinase activity of ADAM 12 even at concentrations 5-fold higher than the enzyme; however, TIMP-3 (500 nm) was an effective inhibitor of ADAM 12 activity (Fig. 2C, lanes 8–15).

ADAM 12 Levels during Disease Progression—117 urine specimens were analyzed, including 46 from healthy age-matched female volunteers and 71 from patients that had been diagnosed with various stages of breast cancer. The majority of samples from cancer patients (Fig. 3A, Table I) were positive for ADAM 12 in contrast to the normal urine samples in which the detection of the two peptides from the prodomain. Although the size of the protein band detected by immunoblot analysis using ADAM 12-specific antibody is most consistent with that of mature ADAM 12-S, the polyclonal antibody rb122 recognizes the cysteine-rich region of ADAM 12, a region of shared homology in both the -L and -S forms. Therefore we cannot rule out the possibility that the band detected is a processed form of ADAM 12-L. Full-length ADAM 12-L has a reported mass of ~120 kDa, whereas the mature (without the propeptide) membrane-anchored form is ~90 kDa (18). Although the ~120-kDa species analyzed by mass spectrometry contained the full-length protein, immunoblot analysis indicated that the major species in urine appears to be the ~68-kDa mature form of the enzyme.

Substrate Specificity of ADAM 12—To test the ability of ADAM 12 to degrade ECM proteins, recombinant ADAM 12-S protein was expressed in COS-7 cells and purified to homogeneity as described previously (Fig. 2A). When assayed by substrate zymography, ADAM 12 could proteolytically degrade gelatin. The purified human ADAM 12-S sample consisted of an ~68-kDa mature form of the enzyme that displayed a gelatinase activity band (Fig. 2B, lane 2). ADAM 12 also degraded fibronectin (Fig. 2B, lane 3) and type IV collagen (Fig. 2B, lane 4) but did not degrade casein (Fig. 2B, lane 5) when assayed by substrate zymography or type I collagen when tested in the radiometric collagenase assay (data not shown). The gelatin-degrading activity of ADAM 12 was used to evaluate the effect of different classes of protease inhibitors on this enzyme. Pure ADAM 12-S was incubated with gelatin (~140-kDa protein band, Fig. 2C, lane 2) at 37 °C for either 2 or 18 h in the absence (Fig. 2C, lanes 3 and 4) or presence of various inhibitors. Disappearance of the ~140-kDa band indicated that ADAM 12 cleaved gelatin in a time-dependent manner (Fig. 2C, lanes 3 and 4). The classical chelators of zinc metalloproteases, EDTA and 1,10-phenanthroline, inhibited gelatin degradation by ADAM 12, whereas the addition of 1 mM Marimastat resulted in partial inhibition of enzyme activity (Fig. 2C, lanes 5–7). The endogenous inhibitors TIMP-1, TIMP-2, and TIMP-4 did not inhibit the gelatinase activity of ADAM 12 even at concentrations 5-fold higher than the enzyme; however, TIMP-3 (500 nm) was an effective inhibitor of ADAM 12 activity (Fig. 2C, lanes 8–15).

ADAM 12 in Breast Cancer
ADAM 12 was detected with significantly lower frequency. The frequency of detection of the ADAM 12 species was higher (94%) in the breast cancer group (67 of 71 urine samples were positive) as compared with the control group (15%) (7 of 46 were positive). Urine samples from the breast cancer patients and the control group also differed in the levels of ADAM 12 protein. An immunoblot analysis of representative urine samples from healthy individuals and patients with progressive stages of breast cancer is presented in Fig. 3.

**Statistical Analysis**—Table I presents the percentage of patients with positive expression of ADAM 12 as well as the median levels for each group. The mean densitometric score for control samples (12 densitometric units) was set as threshold, and percent positive for each cancer group was calculated relative to the control group. As indicated in Fig. 4A, based on comparing percentages, there was a significantly higher proportion of individuals in each breast cancer group with expression of ADAM 12 compared with controls (all p < 0.01). Table II shows the diagnostic characteristics of ADAM 12 as a dichotomous test (based on positive or negative test result) for all patients and for each cancer stage group. For all cancer patients the sensitivity of ADAM 12 based on any level of positive expression is 0.94 (95% confidence interval, 0.86–0.98), and the specificity is 0.61 (28 of 46 controls) (95% confidence interval, 0.46–0.75). Setting a threshold value of 12 densitometric units yielded a much higher specificity of 0.85 (39 of 46 controls) for all cancer patients. Overall accuracy was 0.81 based on 95 of the 117 individuals correctly classified as cases or controls (95% confidence interval, 0.73–0.88). The calculated
likelihood ratio was 2.4, which for a positive test result indicates that the presence of urinary ADAM 12 is associated with 2.4 times greater likelihood that the individual has breast cancer. Median levels of ADAM 12 for all 71 cancer patients and the 46 controls were 37 (interquartile range 14–84) and 0 (range 0–7), respectively (*p* < 0.001, Mann-Whitney *U* test). ROC curves were constructed for the various groups, including all cancer patients and patients with ADH/LCIS, DCIS, IBC, and metastatic disease, to determine the relationship between the true-positive rate (sensitivity) and false-positive rate (1-specificity) for the empirical ADAM 12 levels as a continuous variable (data not shown). All ROC curves indicated excellent discrimination and rose sharply from the 45° diagonal line (i.e. the line of nondiscrimination). The ROC curves based on distinguishing between normal controls and women with IBC and those comparing controls and patients with metastatic disease had the highest area under the curve (0.90 and 0.92, respectively) for ADAM 12 levels greater than 0; the sensitivity for identifying patients with IBC and metastatic disease was perfect (i.e. sensitivity = 1.00).

Logistic regression analysis was used to derive the probability of breast cancer based on ADAM 12 level (Fig. 4B). The empir-
ical levels of ADAM 12 are shown as histograms for cases and controls with the percentage of women having ADAM 12 levels represented on the x axis. Among the 46 controls, 28 (61%) had ADAM 12 levels of 0, whereas only 4 of the 71 cancer patients (6%) had ADAM 12 levels of 0. In addition, 6 controls (13%) had ADAM 12 levels above 20, whereas 46 of the 71 cancer patients (65%) had levels over 20. Logistic regression confirmed a highly significant direct relationship between ADAM 12 level and the probability of cancer (likelihood ratio test = 34.62, \( p < 0.0001 \)). As indicated by the theoretical nonlinear curve in Fig. 4B, the probability of breast cancer is 32% for individuals having an ADAM 12 level of 0 (i.e. no expression) and nearly 50% when levels are between 1 and 20. The model predicts that for ADAM 12 levels between 21 and 40, the probability of breast cancer is 67%, 80% when levels are between 41 and 60, 90% for levels between 61 and 80, 95% for individuals having ADAM 12 levels of 81–100, and 98% when levels exceed 100.

### Table I

| ADAM 12 expression in breast cancer patients and controls |
|---------------------------------------------------------|
| **No. of patients** | Controls | ADH/LCIS | DCIS | IBC | Metastatic |
|---------------------|----------|----------|------|-----|------------|
| 46                  | 13       | 16       | 29   | 13  |
| Age, years          | 41 ± 13  | 52 ± 6   | 57 ± 13 | 59 ± 12 | 55 ± 7 |
| Presence of ADAM 12 | 7 (15%)  | 10 (78%) | 13 (82%) | 25 (86%) | 11 (85%) |
| ADAM 12 level (DU)\(a\) | Mean ± S.E. | 12.4 ± 4.2 | 23.0 ± 6.5 | 47.0 ± 9.8 | 68.4 ± 12.9 | 85.9 ± 17.8 |
| Median              | 0        | 14       | 46   | 43  | 80         |
| IQR\(b\)           | 0–7      | 12–31    | 15–67 | 20–92 | 42–106     |
| Range               | 0–144    | 0–80     | 0–140 | 6–289 | 4–290      |

\(a\) DU, densitometric units.

\(b\) IQR, interquartile range.

**Fig. 4.** Positive expression of ADAM 12 for breast cancer groups and controls. A, urinary ADAM 12 in breast cancer groups and controls. The mean densitometric score for control samples was set as threshold, and percent positive for each cancer group was calculated relative to the control group. Compared with controls, the percentage of individuals with urinary ADAM 12 was significantly higher at each stage of breast cancer ADH/LCIS (78%), DCIS (82%), IBC (86%), and metastatic disease (85%) (\( p < 0.01 \)). B, theoretical curve based on logistic regression analysis illustrating the probability of having breast cancer (as opposed to normal) based on rising level of ADAM 12. The probability curve is superimposed on the percentage of controls and breast cancer patients for specific intervals of ADAM 12 (\( p < 0.0001 \)). The x axis represents intervals of ADAM 12 level, and the y axis corresponds to the actual percentage of patients and controls in each interval.
In this study, a purification protocol designed to isolate and identify previously unidentified protease species in urine from cancer patients followed by mass spectrometric analysis of isolated proteins led to the detection of ADAM 12. MALDI-TOF mass spectrometry identified four distinct peptides that corresponded to different regions of the ADAM 12 amino acid sequence. Three of these peptides originated from the propeptide and catalytic domains of the protease, whereas the fourth peptide corresponded to a region in the C terminus of ADAM 12-L. The detection in urine of a peptide from the cytoplasmic tail of ADAM 12 is surprising given that this is a transmembrane protein the C terminus of which is intracellular, however, it is by no means without precedent. Several groups have recently reported the presence of full-length and/or fragments of membrane-anchored as well as intracellular proteins in urine (19, 20).

ADAM 12 was purified using a gelatin affinity step and because the cognate physiological substrate for this protease had yet to be identified, we tested the substrate specificity of ADAM 12. We found that ADAM 12 can, in fact, degrade gelatin, type IV collagen, and fibronectin but not type I collagen or casein. This is an interesting finding given that proteases that degrade ECM components have been implicated in tumor growth and invasion as a function of their ability to degrade the basement membrane. Several other members of the ADAM family including ADAM 10, ADAM 15, and the closely related snake venom metalloprotease have been reported to have type IV collagen- and gelatin-degrading properties (21–23). Recently, ADAM 13 has been reported to cleave fibronectin (24). In the present study, the gelatinase activity of ADAM 12 was inhibited by EDTA and 1,10-phenanthroline (consistent with the previous observation of Loechel et al. (6) who reported the presence of full-length and/or fragments of membrane-anchored as well as intracellular proteins in urine (19, 20).

The gelatin-degrading activity of ADAM 12, although unaffected by TIMP-1, -2, and -4, showed significant inhibition by TIMP-3. These data are consistent with the previous observation of Loechel et al. (6) who found that the IGFBP-3-cleaving activity of ADAM 12 was similarly inhibited by TIMP-3 but not by TIMP-1 or TIMP-2. Several members of the ADAM family are inhibited by TIMPs. ADAM 10 (25) and ADAM 17 (26) are inhibited by TIMP-1 and TIMP-3 but not TIMP-2, and ADAM 33 is inhibited by TIMP-3 and -4 (27). In addition to being selectively inhibited, these ADAMs also show variable sensitivity to different members of the TIMP family. For example, inhibition dissociation constants (Ki) for ADAM 17 range from 800 nM for TIMP-1 to 2 nM for TIMP-3, whereas Ki values for ADAM 33 have been reported to be 80 and 250 nM for TIMP-3 and TIMP-4, respectively (27). In this study we found that 500 nM TIMP-3 was necessary to detect inhibition of ADAM 12 gelatinase activity. These concentrations are consistent with the wide range of Ki values observed for the other members of the ADAM family despite the fact that they are relatively high in comparison with the concentration required to inhibit MMP activity (28, 29). The ability of ADAM 12 to degrade ECM components and be inhibited by the endogenous metalloprotease inhibitor TIMP-3 is an important finding that may indicate a mechanism of action for this protease in the growth and/or metastasis of tumors similar to that of MMPs. Such a proteolytic activity in vivo would facilitate cancer cell detachment and migration as well as tumor invasion through its surrounding ECM barrier.

Several independent studies have suggested a correlation between ADAM 12 and neoplastic disease. ADAM 12 has been shown to be up-regulated in breast, colon, and lung carcinoma tissue as compared with normal tissue by immunohistochemistry and reverse transcription-PCR (9). Detection of ADAM 12 mRNA has been reported in cell lines derived from a variety of hematological malignancies and giant cell tumors of the bone (2, 3). Recently, Le Pabic et al. (11) have reported an up to 6-fold increase in ADAM 12 mRNA and protein levels in human hepatocellular carcinoma and a correlation with tumor aggressiveness and progression. Although the precise role of this disintegrin metalloprotease in tumor progression is not yet clear, several theories have been suggested. In vitro, the cysteine-rich domain of ADAM 12 supports cell attachment and spreading for a variety of cell types (10) through a syndecan-4-initiated and β1-integrin-dependent process. Carcinoma cells, in contrast, were reported to have distinctly different adhesive properties because of uncoupling of the interaction between syndecans and β1-integrin, thereby influencing tumor cell interactions with the surrounding stroma and affecting the tumor microenvironment. Alternatively, the proteolytic activity of ADAM 12 could modulate the availability of potent growth factors such as IGF (by cleaving IGFBPs) (6) and heparin-binding-EGF (by ectodomain shedding) (8), both of which play a central role in cellular growth, differentiation, and proliferation and are known to contribute to carcinogenesis and carcinoma progression.

Analysis of urine samples from cancer patients at various stages of the disease (Fig. 3) indicated that the level of ADAM 12 in urine differs with both the disease state and stage. In the urine of healthy individuals, ADAM 12 was undetected or present at extremely low levels (Fig. 3A). However, levels of ADAM 12 in urine increased in patients with LCIS, DCIS, and IBC, and the highest levels were observed in metastatic disease. Results of statistical tests based on the densitometric analysis of immunoblots highlight the value of ADAM 12 as a potentially important biomarker in breast cancer. The sensitivity of ADAM 12 is high, ranging from 85% for ADH/LCIS to 100% for IBC and metastatic disease. Using logistic regression analysis, we modeled the probability of breast cancer as a function of ADAM 12 level to show that the predicted probability of breast cancer is ≈80% when the level of ADAM 12 exceeds 40 densitometric units. Statistical diagnostic features of ADAM 12 in differentiating between patients with breast cancer and controls demonstrate with a high degree of confidence that ADAM...
12 will be detected usually at high levels in the urine of individuals with advanced disease. Incurring some false-positive leads while simultaneously correctly classifying nearly all of the cases with breast cancer (only 4 of 71 cases were missed) ultimately provides an excellent tradeoff, particularly when a noninvasive biomarker is used as the basis for testing.

Although further studies are necessary, our data suggest that the diagnostic characteristics of ADAM 12 in differentiating breast cancer patients and controls, in particular with respect to sensitivity, accuracy, and false-negative ratios, are comparable with a number of tumor markers currently in use to monitor disease in cancer patients (30–32). To our knowledge, there are no urinary analyses currently in clinical use as independent predictors of disease status in breast cancer patients. The opportunity to noninvasively detect the presence of breast cancer via the detection of urinary proteins such as ADAM 12 and others has the potential to significantly change the way this cancer is detected and the way that therapeutic efficacy could be monitored. Our data also support the possibility of using urinary ADAM 12 analysis as a “first look” clinical assay and as a means of providing correlative and/or supportive information to physicians in the course of their clinical assessment of patients. Urinary ADAM 12 analysis might also be used in combination with a number of other biomarkers and/or with the standard methods of breast cancer detection, with the ultimate goal being to increase the predictive power of this panel of tests above each individual test used alone.

In this report, we demonstrate that human ADAM 12 possesses proteolytic activity toward various ECM proteins. Zinc chelators, hydroxamate compounds, and TIMP-3 could inhibit this enzymatic activity. This study represents the first biochemical identification of a disintegrin metalloprotease in urine from cancer patients. We show that ADAM 12 can be detected in the urine of breast cancer patients at a frequency higher than that observed for healthy individuals and that its detection is predictive of disease status. The correlation between the levels of ADAM 12 in the urine of breast cancer patients and the stage of disease may reflect the active role played by this protease in tumor progression in vivo. Preliminary studies by our group have indicated that in addition to breast cancer this protein can be detected in urine samples from patients with prostate and bladder cancer (data not shown). Therefore, increased levels of ADAM 12 in urine may be indicative of disease status and tumor progression not only in breast but potentially in other types of cancer. Detailed analysis and statistical evaluations of a larger cohort of samples are currently under way. The detection of urinary ADAM 12 could serve in the development of noninvasive diagnostic tools for cancer diagnosis and therapeutic efficacy.

Acknowledgments—We thank Dr. Jay Harper for critical review of the manuscript and Dr. Mark Puder for the gift of Marimastat. We acknowledge all of our clinical colleagues at the Harvard Medical School Center for Excellence in Women’s Health. We also thank Kristin Gullage for editorial assistance.

REFERENCES

1. Busbaum, J. D., Liu, K. N., Luo, Y., Black, J. L., Stocking, K. L., and Peschon, J. J. (1998) J. Biol. Chem. 273, 27765–27767
2. Chubinskaya, S., Mikhail, R., Deutsch, A., and Tindal, M. H. (2001) J. Histochem. Cytochem. 49, 1165–1176
3. Seale, D. F., and Courtneidge, S. A. (2003) Genes Dev. 17, 7–30
4. Gilpin, B. J., Loechel, F., Mattei, M. G., Engvall, E., Albrechtsen, R., and Wewer, U. M. (1998) J. Biol. Chem. 273, 157–166
5. Loechel, F., Gilpin, B. J., Engvall, E., Albrechtsen, R., and Wewer, U. M. (1998) J. Biol. Chem. 273, 16993–16997
6. Loechel, F., Fox, J. W., Murphy, G., Albrechtsen, R., and Wewer, U. M. (2000) Biochem. Biophys. Res. Commun. 278, 511–515
7. Shi, Z., Xu, W., Loechel, F., Wewer, U. M., and Murphy, L. J. (2000) J. Biol. Chem. 275, 18574–18580
8. Asakura, M., Kitakaze, M., Takashima, S., Liao, Y., Ishikura, F., Yoshinaka, T., Ohmoto, H., Node, K., Yoshino, K., Ishiguro, H., Asanuma, H., Sanada, S., Matsumura, Y., Takeda, H., Beppu, S., Tada, M., Hori, M., and Higashiyama, S. (2002) Nat. Med. 8, 35–40
9. Iba, K., Albrechtsen, R., Gilpin, B. J., Loechel, F., and Wewer, U. M. (1999) Br. J. Pathol. 134, 1499–1503
10. Iba, K., Albrechtsen, R., Gilpin, B., Frohlich, C., Loechel, F., Zolkiewska, A., Ishiguro, K., Kojima, T., Liu, W., Langford, J. K., Sanderson, R. D., Brakebusch, C., Passler, R., and Wewer, U. M. (2000) J. Cell Biol. 149, 1143–1155
11. Le Pabic, H., Bonnier, D., Wewer, U. M., Coutand, A., Musso, O., Baffet, G., Clement, B., and Theret, N. (2003) Hepatology 37, 1056–1066
12. Lochter, A., Sternlicht, M. D., Werb, Z., and Bissell, M. J. (1998) Ann. N. Y. Acad. Sci. 857, 180–193
13. Kleiner, D. E., and Stetler-Stevenson, W. G. (1999) Cancer Chemother. Pharmacol. 43, 342–351
14. Moses, M. A., Wiederschain, D., Loughlin, K. R., Zurakowski, D., Lamb, C. C., and Freeman, M. R. (1998) Cancer Res. 58, 1395–1399
15. Yan, L., Borregaard, N., Kjeldsen, L., and Moses, M. A. (2001) J. Biol. Chem. 276, 37258–37265
16. Moses, M. A., Sudhalter, J., and Langer, R. (1990) Science 248, 1408–1410
17. Breslow, N. E., and Day, N. E. (1980) Statistical Methods in Cancer Research: The Analysis of Case-control Studies, Vol. 1, pp. 192–224, International Agency for Research on Cancer, Lyon, France
18. Cao, Y., Kang, Q., Zhao, Z., and Zolkiewska, A. (2002) J. Biol. Chem. 277, 27903–27911
19. Pieper, R., Gatlin, C. L., McGrath, A. M., Makusky, A. J., Mondal, M., Seonarain, M., Field, E., Schatz, C. R., Estock, M. A., Ahmed, N., Anderson, N. G., and Steiner, S. (2004) Proteomics 4, 1159–1174
20. Thongsonkerd, V., McLeish, K. R., Arthur, J. M., and Klein, J. B. (2002) Kidney Int. 62, 1461–1469
21. Millichip, M. I., Dallas, D. J., Wu, E., Dale, S., and Mckie, N. (1998) Biochem. Biophys. Res. Commun. 245, 594–598
22. Martin, J., Eynton, L. V., Davies, M., Williams, J. D., and Steadman, R. (2002) J. Biol. Chem. 277, 33683–33689
23. Shannon, J. D., Baranova, E. N., Bjarnason, J. B., and Fox, J. W. (1989) J. Biol. Chem. 264, 11575–11583
24. Alfandari, D., Cousin, H., Gaultier, A., Smith, K. White, J. M., Darribere, T., and Del Simone, D. W. (2001) Curr. Biol. 11, 918–930
25. Amour, A., Knight, C. G., Webster, A., Slcombe, P. M., Stephens, P. E., Knauper, V., Docherty, A. J., and Murphy, G. (2006) FEBS Lett. 573, 275–279
26. Amour, A., Slcombe, P. M., Webster, A., Butler, M., Knight, C. G., Smith, B. J., Stephens, P. E., Shelley, C., Hutton, M., Knauper, V., Docherty, A. J., and Murphy, G. (1998) FEBS Lett. 435, 39–44
27. Zhou, J., Zhu, F., Liu, J., Wang, Z., Zhang, R., Garlisi, C. G., Liu, Y.-H., Wang, S., Shah, H., Wan, Y., and Umland, S. P. (2004) J. Biol. Chem. 279, 9818–9830
28. Visse, R., and Nagase, H. (2003) Curr. Res. 92, 827–839
29. Fernández, C. A., Butterfield, C., Jackson, G., and Moses, M. A. (2003) J. Biol. Chem. 278, 40989–40995
30. Prestigiacomo, A. F., Lilja, H., Pettersson, K., Wolfert, R. L., and Staney, T. A. (1996) J. Urol. 156, 350–354
31. Moertel, C. G., Fleming, T. R., Marden, J. S., Hailer, D. G., Laurie, J. A., and Tangen, C. (1993) J. Am. Med. Assoc. 270, 943–947
32. Berk, J. S., and Bast, R. C., Jr. (1995) Cancer (Phila.) 76, 2092–2096