Type IV Collagenase/Gelatinase (MMP-2) Is Not Increased in Plasma of Patients with Cancer

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Abstract

We have developed a sensitive and specific sandwich-type enzyme-linked immunosorbent assay to detect M, 72,000 type IV collagenase [matrix metalloproteinase 2 (MMP-2)] in human plasma. As a result of the linkage between MMP-2 production by cancer cells and the metastatic phenotype, we undertook this study to compare plasma MMP-2 levels in healthy individuals, patients with various types of cancer, and hospitalized patients with chronic diseases other than cancer. The results demonstrate that MMP-2 levels are not increased in cancer patients regardless of the extent of disseminated malignancy. In an effort to explain this data, we compared MMP-2 secretion by human umbilical vein endothelial cells and lung cancer cells passaged as cell lines. Endothelial cells secreted higher levels of MMP-2 than did lung cancer cells propagated

Materials and Methods

Commercial Reagents. The ELISA for the measurement of MMP-2 in plasma has recently been described in detail (8). Polyclonal antibodies to MMP-2 were produced in rabbits with native human MMP-2 purified from human A2058 melanoma cells (9). Polyclonal antibodies to MMP-2 were affinity purified with MMP-2 bound to Affigel 10 (BioRad, Richmond, CA). Monoclonal antibodies were produced in BALB/c mice (10) by the injection of human MMP-2. Lymph node cells were fused with murine myeloma cells, and positive hybridomas were cloned and recloned by limiting dilution. Ascites was produced by i.p. injection of hybridoma cells into BALB/c mice. IgG antibodies were isolated by protein A-Sepharose

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3 The abbreviations used are: MMP, matrix metalloproteinase; ELISA, enzyme-linked immunosorbent assay; TIMP, tissue inhibitor of metalloproteinases; FCS, fetal calf serum; H, heavy chain; L, light chain.

have described a close correlation between the secretion of MMPs by cancer cells, especially MMP-2 (M, 72,000 type IV collagenase/gelatinase), and the invasive and metastatic potential of these cells in experimental models (2–7). These studies of tumor-conditioned media used radiolabeled substrate (gelatin or type IV collagen) degradation assays to quantify the combined activity of MMP-2, MMP-3 (stromelysin), and MMP-9 (M, 92,000 type IV collagenase/gelatinase) or gelatin zymograms to qualitatively estimate the relative amount of each MMP as individual bands of gelatin lysis.

We recently developed a sandwich-type ELISA for measurement of MMP-2 in human plasma which does not cross-react with other MMPs and is many times more sensitive than standard bioassays (8). Plasma MMP-2 levels were found to be significantly increased in late pregnancy, which is consistent with the extensive amount of connective tissue remodeling occurring in that condition. Based on evidence that invasive and metastatic human cancer cells actively produce MMP-2, we initiated this study to determine whether plasma MMP-2 measurements could serve as a tumor marker for aggressive cancers. We report herein the results of a plasma assay of MMP-2 in 230 patients with different types of cancers.

Sandwich ELISA for the Detection of Human MMP-2. Ninety-six-well microtiter plates were coated with rabbit polyclonal antibodies (2.5 mg protein/well) to human MMP-2. The unbound anti-MMP-2 was removed, and bovine serum albumin was added to block excess binding sites on the wells. The plates were then washed. Human plasma samples and purified human MMP-2 standards were diluted and added to the wells. The wells were washed, and murine monoclonal anti-human MMP-2 antibodies (72K-VB3, 0.3 mg protein/well) were added. The plates were washed, and biotin-labeled goat antibodies to mouse IgG, IgA, and IgM (H + L) were added. The plates were washed, and alkaline phosphatase con-
jugated to streptavidin was added to each well. After incubation, the wells were washed, and p-nitrophenyl phosphate (1 mg/ml) substrate was added. The plates were read at A405 in a microplate autoreader (Biolek EL 309; BioTek, Winooski, VT). Quantification of MMP-2 was made by extrapolation from a log-log linear regression curve, using varying concentrations of purified MMP-2 as the standard. (For details see Ref. 8.)

**Calibration of Assay.** The ELISA for MMP-2 is linear on a log-log scale for protein concentrations between 2 and 2000 ng/ml using human MMP-2 purified from A2058 melanoma cells; similarly, serial dilutions of human plasma were linear within these protein concentrations (8). The intraassay and interassay precision of the MMP-2 ELISA was between 8 and 13% (coefficient of variation) (8). No cross-reactivity of the MMP-2 ELISA with MMP-1, MMP-3, MMP-9, TIMP-1, or TIMP-2 was noted. This plasma assay is capable of detecting free latent and free activated MMP-2, as well as MMP-2 complexed with TIMP-1 (8).

**Concentrations of MMP-2 in Human Plasma.** The distribution of plasma MMP-2 values for healthy individuals fit a Gaussian distribution. The mean MMP-2 concentration for men [489 ± 182 (SD) ng/ml] was higher than that...
The effect of prior therapy on MMP-2 was assessed by dividing cancer patients into eight groups: no treatment; chemotherapy only; radiation therapy only; surgery only; or the four possible combinations of treatments. No significant differences in MMP-2 were noted between groups. Comparison of patients with high MMP-2 levels within each cancer group revealed no identifiable differences in terms of site or extent of disease, pathologic classification, or response to treatment.

MMP-2 Secretion of Endothelial Cells and Cancer Cells.

The content of MMP-2 in endothelial cell-conditioned medium containing 2% FCS at 2, 4, and 24 h was 9.2, 18.9, and 70.0 ng/ml, respectively, with little discernible difference noted by substitution of 20% FCS in the media (data not shown). In two other experiments using different endothelial cell harvests, the concentration of MMP-2 in 24-h conditioned media varied between 430 and 625 ng/ml. The ELISA for human MMP-2 did not cross-react with bovine MMP-2 in lethal cell serum. These data suggest that the umbilical vein source and the incubation conditions are important determinants in modulating the secretory rate of MMP-2 by endothelial cells.

The content of MMP-2 in conditioned media from human lung cancer cells cultivated in 10% FCS for 24 h varied from a low of 4–5 ng/ml for the nonmetastatic cell lines (HU281 and SKLU-1) to a high of 9–17 ng/ml for the cell lines capable of producing lung metastases (A549 and Calu-1) in nude mice. The content of MMP-2 in media (containing 10% FCS) conditioned by Krev-1 revertant Calu cell lines was 115–120 ng/ml. MMP-2 secretion by cancer cells in media lacking FCS was approxi-
mately 10-fold less than with cells cultivated in the presence of FCS (13).

Discussion
In this report we have used a sensitive and specific immunoassay to measure MMP-2 in human plasma in healthy individuals and in patients with a variety of chronic diseases. This ELISA detects both latent and activated MMP-2 in either the free state or circulating as complexes with TIMP-2 (8). Previously we reported increased plasma levels of MMP-2 in the second half of pregnancy, which is consistent with the extensive amount of connective tissue remodeling in pregnancy (8). Rajabi et al. (14) reported that plasma levels of MMP-1 (interstitial collagenase) were increased in pregnancy, but only in the few days preceding delivery.

Based on the considerable amount of evidence indicating increased production of MMP-2 by invasive cancer cells, we initiated this study expecting to find high levels of plasma MMP-2 in patients with aggressive cancer. However, our results demonstrated that, regardless of the type or clinical stage of cancer, less than 15% of the cancer patients evaluated had plasma MMP-2 levels above the normal range (0–940 ng/ml). Similar elevations in plasma MMP-2 were noted in some hospitalized patients with other serious illnesses (end-stage liver, kidney, lung, and heart disease), thus indicating that, in general, MMP-2 is not a useful plasma marker for cancer. However, it is possible that a subgroup of cancer patients with elevated MMP-2 may be identified. It is of interest to note that plasma concentrations of MMP-2 remained relatively constant over time for each individual regardless of whether the level was high or normal. This suggests the influence of homeostatic mechanisms regulating plasma metalloproteinase concentrations in individual patients.

To gain insight into the production of MMP-2 by normal cells involved in tissue remodeling as compared to cancer cells, we compared MMP-2 secretion by human endothelial cells versus lung cancer cells. In these experiments, umbilical vein endothelial cells cultivated in vitro secreted higher levels of MMP-2 than did lung cancer cells propagated in long-term culture. Likewise, MMP-2 production by endothelial cells exceeded enzyme production by 11 human breast cancer cell lines cultivated in vitro. Other studies have also demonstrated that various types of endothelial cells secrete large amounts of MMP-2 and TIMPs; secretion is directed both apically (representing secretion into the bloodstream) and basally and is under physiological control (15–17). A limitation to this type of experiment is the assumption that MMP secretion in vitro by second-passage endothelial cells from umbilical veins or cancer cell lines passaged in vitro bears a close resemblance to the secretion of counterpart cell types in vivo. Likewise, it is not possible to cultivate these different cell types under identical conditions and obtain optimal cell growth. Other reports have shown that gelatinase/type IV collagenase production by normal fetal uroepithelial cells exceeded production by invasive bladder cancer cell lines cultivated in vitro. However, the neoplastic cells were reported to be less responsive to regulatory signals. Allen et al. (18) raised the possibility that high levels of gelatinase production may be characteristic of fetal cells but not their adult counterparts.

Considering the vast number of endothelial cells in the body, the large number of mesenchymal cells involved in physiological tissue remodeling, and the potential for high levels of MMP-2 secretion by these cells, it is reasonable to propose that production of MMP-2 by even a bulky tumor may not contribute sufficiently to the plasma pool of MMP to permit enzyme detection above basal levels. Other explanations for the lack of correlation of plasma MMP-2 levels with advanced cancer are: (a) high levels of MMP-2 secreted by cancer cells are inactivated, removed locally, and do not leach into the bloodstream; (b) MMP-2 may not be secreted in vivo in increased amounts by cancer cells, yet may function locally as a membrane-bound proteinase (19); or (c) MMP-2 levels might be increased only in some patients, with MMP-3, MMP-9, serine proteinases, or cysteine proteinases being the predominant proteinases in other cancer patients.

Elevated plasma levels of type IV collagenolytic activity (using the less sensitive substrate degradation assay) were reported by Hashimoto et al. (21) in a subset of patients with hepatocellular carcinoma and portal vein invasion. Likewise, elevated levels of serum type IV collagenolytic activity have been described in an experimental model of cancer (22). The discrepancy between our findings and the latter report may be explained by its use of serum for bioassay, which leads to falsely elevated levels of M, 92,000 type IV collagenase following the degradation of granulocytes during blood clotting. The development of a laboratory test for screening, diagnosis, and/or management of patients with the more common types of cancer remains an elusive goal. In concept, the identification in plasma of increased levels of proteins produced by cancer cells that are necessary for invasion and metastasis is a logical and feasible objective. In future studies it will be important to select plasma tumor markers that are not released by endothelial cells or leukocytes which have immediate access to the biological fluid (blood) being assayed and thereby produce an imbalance in the relative amount of the plasma protein being assayed as compared to proteins secreted by solid tumors, which presumably remain primarily in interstitial tissues.

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