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# Activation of Pro-uPA Is Critical for Initial Escape from the Primary Tumor and Hematogenous Dissemination of Human Carcinoma Cells[1,2]

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## Abstract

Urokinase-type plasminogen activator (uPA) and plasmin have long been implicated in cancer progression. However, the precise contributions of the uPA/plasmin system to specific steps involved in cancer cell dissemination have not been fully established. Herein, we have used a highly disseminating variant of the human PC-3 prostate carcinoma cell line, PC-hi/diss, as a prototype of aggressive carcinomas to investigate the mechanisms whereby pro-uPA activation and uPA-generated plasmin functionally contribute to specific stages of metastasis. The PC-hi/diss cells secrete and activate significant amounts of pro-uPA, leading to efficient generation of plasmin in solution and at the cell surface. In a mouse orthotopic xenograft model, treatment with the specific pro-uPA activation–blocking antibody mAb-112 significantly inhibited local invasion and distant metastasis of the PC-hi/diss cells. To mechanistically examine the uPA/plasmin–mediated aspects of tumor cell dissemination, the anti–pro-uPA mAb-112 and the potent serine protease inhibitor, aprotinin, were used in parallel in a number of *in vivo* assays modeling various rate-limiting steps in early metastatic spread. Our findings demonstrate that, by generating plasmin, activated tumor-derived uPA facilitates early stages of PC-hi/diss dissemination, specifically the escape from the primary tumor and tumor cell intravasation. Moreover, through a series of *in vitro* and *in vivo* analyses, we suggest that PC-hi/diss–invasive escape and dissemination may be enhanced by cleavage of stromal fibronectin by uPA-generated plasmin. Together, our findings point to inhibition of pro-uPA activation at the apex of the uPA/plasmin cascade as a therapy-valid approach to control onset of tumor escape and ensuing metastatic spread.

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## Introduction

Increased levels and activity of proteolytic enzymes have been linked to enhanced motility, invasion, and metastasis of tumor cells [1,2]. Specifically, the serine protease urokinase-type plasminogen activator (uPA) is often elevated in aggressive cancers, especially in prostate cancer [3–6]. In normal tissues, uPA is tightly regulated at the level of proenzyme activation as well as at the level of enzyme activity. However, during cancer invasion and metastasis this tight control of uPA system is dysregulated.

The uPA molecule is secreted as an inactive, single-chain zymogen, pro-uPA, which must be proteolytically converted into an active enzyme before it can exert its major biologic function, that is, convert plasminogen to the active plasmin. In a reciprocal fashion, plasmin activates the single-chain pro-uPA through hydrolysis of the $Lys_{158}$-$Ile_{159}$ bond, yielding two, A and B chains, which remain covalently

Abbreviations: CAM, chorioallantoic membrane; CM, conditioned medium; DMEM, Dulbecco modified Eagle medium; ECM, extracellular matrix; LCA, *Lens culinaris* agglutinin; mAb, monoclonal antibody; uPA, urokinase-type plasminogen activator; uPAR, urokinase-type plasminogen activator receptor

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linked by a disulfide bond [7–10]. Although a secreted protease, uPA can be tethered to the cell surface through binding of its growth factor–like domain to the GPI-anchored cell surface receptor, uPAR [11,12]. Both pro-uPA and activated uPA bind uPAR with similar affinities; however, cell surface–bound uPA is significantly more potent in the catalytic conversion of plasminogen to plasmin [13]. Plasmin (ogen) also can be localized to the cell surface by binding to C-terminal lysine residues of several membrane proteins, including annexin II and Plg-RKT [14,15]. Furthermore, cell surface–bound plasmin is protected from inhibition by circulating inhibitors, thereby enhancing its cell-associated functions [16]. The pericellular localization of both uPA and plasmin activity is believed to facilitate cell invasion during wound healing and tumor progression. Once activated by uPA, plasmin has a broad substrate repertoire. Whereas the canonical function of active plasmin is fibrin clot lysis, plasmin can also cleave several noncollagenous components of the extracellular matrix (ECM), such as fibronectin and laminin [17–19]. In addition, plasmin is involved in proteolytic activation of additional proteases, for example, matrix metalloprotease (MMP) zymogens, including pro–MMP-1 and pro–MMP-3 [20,21].

Extensive evidence implicates elevated expression of uPA in malignant cells and the activity of uPA/plasmin in overall tumor progression and metastasis [7,9,22,23]. Inhibition of uPA activity by antibody and small molecule inhibitors has been shown to diminish tumor growth, angiogenesis, and metastasis in several model systems [6,10,24]. In addition, small interfering RNA and short hairpin RNA knockdown of uPA expression in prostate carcinoma cells diminished their invasion *in vitro* and tumor growth, angiogenesis, and dissemination to the lung in an orthotopic mouse model [25,26], and significantly reduced bone tumor burden and bone destruction in a bone metastasis xenograft model [27]. Furthermore, small interfering RNA targeting of the uPA promoter also decreased tumor growth, angiogenesis, and metastasis of prostate carcinoma cells [28]. However, because of the coordinate inhibitory effects on tumor growth, it was difficult to discriminate in these studies direct inhibitory effects of uPA RNA silencing on tumor metastasis from indirect consequences of reduced primary tumor size.

Some of the most compelling data implicating the plasminogen activation cascade in tumor progression come from transgenic mice deficient in various components of the PA system. Interestingly, most phenotypes associated with plasminogen deficiency were not observed in mice with an additional deficiency in fibrinogen [29]. However, in the MMTV-PymT model of breast carcinogenesis combined with genetic deficiency in plasminogen, spontaneous metastasis was significantly reduced without major effects on primary tumor growth [30]. Similarly, uPA deficiency resulted in greatly reduced levels of lung metastasis from MMTV-PymT–induced mammary carcinomas without affecting their growth [31]. Therefore, the single deficiency in uPA was sufficient to reduce metastasis, affirming that uPA is the major plasminogen activator functioning during tumor dissemination.

Despite all the evidence indicating that the uPA/plasmin system plays a critical role in tumor metastasis, the functional contributions of pro-uPA activation and uPA activity in the individual steps involved in the metastatic cascade remain to be elucidated. Using a chemical proteomic approach and function-blocking antibodies, our laboratory has previously identified activation of pro-uPA as a likely key step in the intravasation and metastatic spread of a highly disseminating variant of human HT-1080 fibrosarcoma [32,33]. We have recently isolated a pair of PC-3 prostate carcinoma cell variants with high and low dissemination capacities, namely, PC-hi/diss and PC-lo/diss, and showed that these cell variants exhibit a differential in uPA secretion and uPA activation [34].

In the present study, we sought to identify those individual events during metastatic progression that involve uPA activation. The function-blocking monoclonal antibody (mAb) 112 that uniquely prevents activation of the human pro-uPA zymogen has been used to address the contributory roles of tumor-produced uPA and uPA-generated plasmin in specific processes involved in cancer metastasis. Our findings from several quantitative *in vitro* and *in vivo* models demonstrate that uPA activation and plasmin activity are critically involved in early metastatic events, particularly in the invasive escape from the primary tumor, which we monitored in a newly developed *in vivo* model of tumor cell escape. Furthermore, toward defining the molecules mediating the effects of the uPA/plasmin activation cascade, we demonstrate that cleavage of matrix fibronectin by tumor uPA-generated plasmin results in enhanced migration-inducing capacity of the cleaved fibronectin. Together, our findings underscore a mechanism, by which elevated levels of uPA expression, activation, and activity render cancer cells with a high malignant potential due to enhanced invasive escape from primary tumors along the plasmin-modified ECM.

## Materials and Methods

### *Tissue Culture*

Dissemination variants derived from the human PC-3 prostate carcinoma cell line, PC-hi/diss and PC-lo/diss [34], were maintained in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal calf serum and 10 μg/ml gentamicin (D10).

### *Antibodies*

The following polyclonal and mAbs were used: anti–human pro-uPA mAb-112 [33], anti–human CD44 mAb 29-7 [34], anti–human $\beta_1$ integrin mAb P5D2 (R&D Systems, Minneapolis, MN) and $\alpha_5$ integrin mAb P1D6 (Chemicon, Temecula, CA), rabbit anti-CD31 (Abcam, Cambridge, United Kingdom), and murine control IgG (Jackson ImmunoResearch, West Grove, PA). The supernatants from hybridomas B3/D6 (anti–avian fibronectin) and "31 or 31-2" (anti-avian laminin), developed by D.M. Fambrough, were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the National Institute of Child Health and Human Development and maintained by the Department of Biology at the University of Iowa (Iowa City, IA).

### *Orthotopic Prostate Tumor Model*

Orthotopic implantations of PC-hi/diss cells into the prostates of immunodeficient mice were performed as described in Conn et al. [34]. Briefly, 8- to 9-week-old NOD-SCID mice were purchased from the TSRI breeding colony and maintained under the guidelines of the TSRI Institutional Animal Care and Use Committee. Mice were anesthetized with ketamine/xylazine and $2 \times 10^6$ of firefly luciferase–labeled PC-hi/diss cells in 30 μl of SF-DMEM were implanted into the anterior prostates through an incision in the lower abdomen. After 7 days, the mice were noninvasively imaged by IVIS (Caliper Life Sciences, Mountain View, CA) and separated into two treatment groups containing mice with tumors of similar sizes. Mice received intraperitoneal injections of 150 μg of mAb-112 or control IgG in 150 μl of phosphate-buffered saline (PBS) every 4 to 5 days. Mice were imaged again on day 27 and sacrificed at day 28. Exposed primary tumors were photographed, excised, and weighed, and internal organs were harvested and frozen for *Alu* quantitative polymerase chain reaction (qPCR) analysis. Three separate experiments, involving a total of 14 mice treated with control IgG and 11 mice treated with mAb-112, were performed.