Differential Release of Active Proteinase Inhibitors by Two Rat Mammary Adenocarcinoma Variants Possessing Different Metastatic Potentials

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ABSTRACT

The ability of tumor cells to express elevated levels of proteinases capable of degrading tissue matrix and basement membrane components in vitro has been correlated to their invasive and metastatic potential. Many in vitro invasion assays have been performed either in the presence of serum or with tumor cells that had been previously grown in serum. Since serum contains large amounts of active proteinase inhibitors, their presence could complicate interpretations. We have, therefore, attempted to measure the amounts of serum proteinase inhibitors released into culture medium by two rat mammary adenocarcinoma metastatic variants selected in vitro for serum-independent growth and differing in their in vivo metastatic behavior. Concentrated spent media (CSM) derived from cultures of poorly metastatic MTLn2(T42D) and highly metastatic MTLn3(T17D) tumor cells, grown in the presence and absence of fetal bovine serum (FBS) for 20–24 h, were compared for the presence of serum proteinase inhibitors capable of inactivating α-chymotrypsin. Our results show that when MTLn2(T42D) and MTLn3(T17D) tumor cells were exposed to FBS, the CSM of MTLn2(T42D) exhibited nearly 5-fold greater amounts of active proteinase inhibitors than that of MTLn3(T17D). The amount of proteinase inhibitory activity detected in the CSM of tumor cells not exposed to FBS was not eliminated but declined by 82% and 37% for MTLn2(T42D) and MTLn3(T17D), respectively. Analysis for enzyme-inhibitor (E-I) complex formation by nonreducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by autoradiography confirmed the kinetic results and revealed that the major inhibitor present in CSM/FBS of both variants forms a heat- and sodium dodecyl sulfate-stable E-I complex with an apparent molecular weight of approximately 79,000, identical to that formed when FBS or purified α-proteinase inhibitor is incubated with [125I]α-chymotrypsin. E-I complexes with apparent molecular weights of 44,000 and 50,000 were formed from CSM/bovine serum albumin of MTLn2(T17D) and MTLn2(T42D), respectively, that were not detected when [125I]-α-chymotrypsin was incubated with bovine serum albumin. We infer from these observations that, in culture, poorly metastatic MTLn2(T42D) tumor cells, as compared to their highly metastatic MTLn3(T17D) counterparts, exhibit an increased capacity to retain and subsequently release significantly greater amounts of serum-derived active proteinase inhibitors. Moreover, the detection of proteinase activity by kinetic analysis and E-I complexes by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography in CSM prepared from cultures not exposed to FBS indicates that both variants have the capacity to produce their own inhibitors. This study calls attention to the possible involvement of tumor cell-produced and serum-derived proteinase inhibitors in cancer invasion and metastasis.

INTRODUCTION

Solid malignancies are often characterized by their ability to invade surrounding normal tissues and metastasize to new sites. However, the mere potential for tumor cells to translocate to distant sites does not in itself ensure that successful metastases will develop (1). The immune status of the host, as well as the ability of the tumor cells to establish the necessary conditions required for successful growth at the new site, will determine whether clinically apparent metastatic lesions will emerge (2, 3). The capacity for malignant cells to invade local tissues is considered to be an important aspect of the metastatic process. Much of our understanding of tumor cell and host mechanisms operating during invasion stems from in vitro and in vivo observations of experimental and natural tumors (4). Among other factors associated with the invasive tumor cell, such as adhesion and motility (5), studies have shown that highly invasive and metastatic tumor cells can express higher levels of a variety of tissue degrading enzymes, i.e., collagenases, heparanases, elastases, cathepsins, and serine proteinases, than their poorly metastatic counterparts (6, 7). However, increased expression of proteolytic activity by metastatic tumor cells could also result from a lack or decreased production of endogenous proteinase inhibitors (8–10). It is thought that the process of tumor invasion can also be facilitated by proteinases released by inflammatory cells which often accumulate at sites of tumor invasion and tissue destruction (11–13). For example, host cells, such as infiltrating macrophages, can produce not only a variety of proteinases, but also several proteinase inhibitors including α1-proteinase inhibitor (14–16), one of the major serine proteinase inhibitors found in both animal and human serum (17). We, therefore, believe, as do other investigators (18–20), that degradation of extracellular matrix components may be, in part, regulated by proteinase inhibitors originating from tumor cells and host cells, as well as those inhibitors in serum that are able to effectively diffuse into tissues. Although many in vitro matrix degradation studies have been performed in media containing serum, other studies have measured the invasive capacity of tumor or normal cells by either previously culturing the cells in the presence of serum or completely eliminating the requirement for serum in their assays, thus avoiding any possible interference by serum components (21, 22). Among the 3 major types of proteinases (i.e., serine, cysteine, and metalloproteinases) thought to be involved in the process of invasion and metastasis, this report focuses on the detection of serine proteinase inhibitors by using two metastatic variants in culture. In order to determine the origin and relative amounts of active inhibitors, we have obtained, by in vitro selection, two isoegenous rat mammary adenocarcinoma variants capable of indefinite serum-independent proliferation in culture and differing in their metastatic and lung colonizing potentials when assayed in vivo. We have compared their in vivo metastatic behavior with their ability to express active serine proteinase inhibitors in vitro when cultured in the presence and absence of serum.

MATERIALS AND METHODS

Animals. Female Fischer 344 rats 6–9 weeks old were obtained from Charles River Breeding Laboratories, Inc. (Wilmington, MA). The

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animals were confirmed to be free of Sendai virus, rat coronavirus, rat sialodacryoadenitis virus, pneumonia virus of mice, Kilham rat virus, lymphocyte choriomeningitis virus, reovirus 3, and Mycoplasma pneumoniae, by the vendor and Roche Laboratory Animal Resources Diagnostic Laboratory. The animals were maintained on a diet of rodent laboratory chow (Purina Mills, Inc.) and tap water ad libitum.

Tumor Cells. The derivation of rat 13762NF mammary adenocarcinoma tumor cell variants MTLn2 and MTLn3 used in these studies has been previously described in detail (23). Briefly, tumor cell clones MTLn2 and MTLn3 were derived by in vitro cloning of tissue cultures established from pulmonary tumor lesions resulting from the spontaneous metastasis of the 13762NF parent tumor, which had been implanted s.c. in the mammary fat pads of syngeneic female Fischer 344 rats. Subsequent in vivo analysis of these tumor cell clones, for their ability to spontaneously metastasize from the mammary fat pads or to colonize the lungs of animals after i.v. injection, revealed that MTLn3 produced significantly more lung metastases than MTLn2 (23, 24). In order to determine whether the difference in metastatic behavior observed between MTLn2 and MTLn3 might be related to the expression of tumor cell-derived proteinase inhibitors, it was necessary to adapt and grow the tumor cells in the absence of FBS. This would allow for the detection of active inhibitors produced by the tumor cells without interference by proteinase inhibitors known to be present in serum (17).

In Vitro Selection of MTLn2 and MTLn3 Tumor Cell Variants for Serum-free Growth. MTLn2 tissue culture passage 42 [MTLn2(T42)] and MTLn3 tissue culture passage 17 [MTLn3(T17)] were routinely cultured to confluence in T-75 flasks (Corning) containing RPMI 1640 (Gibco) supplemented with 10% heat-inactivated FBS (BioCell). Serum-free Growth. MTLn2 tissue culture passage 42 [MTLn2(T42)]—and grow the tumor cells in the absence of FBS. This would allow for the detection of active inhibitors produced by the tumor cells without interference by proteinase inhibitors known to be present in serum (17).

To examine whether these metastatic variants were also able to bind and subsequently release active proteinase inhibitors derived from serum, an additional 20–24 h of incubation in the presence of RPMI/FBS (15 ml/culture) was included. The cultures were then washed 3 times with 15 ml of PBS and further incubated for 24 h in RPMI 1640 (i.e., final volume of 6 ml/culture) devoid of any protein supplement. Culture supernatants were collected, centrifuged at 20,000 × g for 30 min and concentrated at 4°C to volumes of 300–600 μl by using Amicon PM10 membrane (exclusion limit, M, <10,000) (CSM). CSM from FBS-exposed (CSM/FBS) and non-FBS-exposed (CSM/BSA) cultures were assayed for the presence of proteinase inhibitors by using bovine α-chymotrypsin (EC 3.4.21.1; Worthington Biochemical Corp.).

Enzyme Inhibition Assay. Various amounts of CSM/FBS and CSM/BSA, obtained from cultures of MTLn2(T42D) and MTLn3(T17D), were preincubated for 10 min at 37°C with 19.2 milliunits of α-Chy (represents 100 ng of active enzyme determined by the hydrolysis of BTEE as described below) in a reaction volume of 0.186 ml containing 100 μg of BSA in RPMI 1640, pH 7.2. Control reactions included indicated amounts of BSA, FBS, or α-PI (human α-1-antitrypsin; Sigma Chemical Co.). Following the preincubation period, the reaction mixtures were immediately assayed for residual enzyme activity. The assay for α-Chy activity was a modified version of the Hummel assay (26) and measured the increase in absorbance at 259 nm resulting from the hydrolysis of 500 μM BTEE in a buffer containing 10% dimethyl sulfoxide, 1.0 mM CaCl2, and 100 mM Tris-HCl, pH 7.2, at 37°C. The amount of protein in CSM/FBS, CSM/BSA, and BSA was determined by the Bio-Rad protein assay (Bio-Rad Laboratories).

Detection of Enzyme-Inhibitor(s) Complex Formation by Nonreducing SDS-PAGE and Autoradiography. α-Chy was radioiodinated by the chloramine-T method. The reaction was allowed to proceed for 15 s, in a reaction volume of 60 μl containing 500 μCi of Na125I (carrier free; Amersham), 250 μg of α-Chy, and 30 μl of 300 μg/ml of chloramine-T prepared in 0.5 μm sodium phosphate, pH 7.2. The reaction was stopped by the addition of 100 μl of a saturated solution of tyrosine in PBS and immediately applied to a 10-ml column of Sephadex G-25 that had been equilibrated with 0.001 N HCl. This procedure typically results in [α125I]Chy that retains greater than 70% activity as determined in the BTEE assay and a specific radioactivity of 1.0–1.5 × 106 cpm/μg protein. The labeled enzyme was routinely stored at 4°C in 0.001 N HCl for up to 2 weeks without any detectable loss of activity.

CSM/BSA and CSM/FBS (each containing 40 μg of cell-released protein) prepared from cultures of MTLn2(T42D) and MTLn3(T17D), 40 μg of BSA, 4.0 μg of FBS protein, and 10.0 μg of α-PI were each incubated with 50,000 cpm of [α125I]Chy (representing 31.6 ng of active enzyme) for 10 min at 37°C in a total volume of 63.0 μl. The samples were then heated at 100°C for 3 min in electrophoresis sample buffer, containing 2% SDS but lacking β-mercaptoethanol, and loaded onto a 7.5% SDS-polyacrylamide gel containing a 3% stacking gel (27). Following electrophoresis, the gels were stained with Coomassie blue,
and reached a final density of $2.5 \times 10^3$ cells/cm$^2$ and $2.7 \times 10^5$ cells/cm$^2$, respectively. Although the plating efficiency for MTLn3(T17D) tumor cells was slightly less in RPMI/BSA (i.e., 80%) than in RPMI/FBS, these results indicate that both tumor cell variants grow well in the absence of FBS.

Detection of Tumor Cell-released Proteinase Inhibitors Capable of Inactivating $\alpha$-Chymotrypsin. The inhibition of $\alpha$-Chy activity by varying amounts of CSM/FBS and CSM/BSA obtained from cultures of MTLn2(T42D) and MTLn3(T17D) (as described in “Materials and Methods”) is illustrated in Fig. 2. Our results show that when MTLn2(T42D) and MTLn3(T17D) tumor cells were cultured for a period of 20–24 h in RPMI 1640 containing 10% FBS, followed by an additional 20–24 h of growth in medium devoid of any protein supplementation, the CSM were capable of inhibiting the activity of $\alpha$-Chy in a dose-dependent manner. The amount of cell-released protein in CSM/FBS required to give an $I_{50}$ value of 19.2 milliunits of $\alpha$-Chy activity for MTLn2(T42D) and MTLn3(T17D) cultures was 24.5 and 117.0 ng, respectively (Table 1). In order to achieve 50% inhibition of $\alpha$-Chy from the CSM prepared from cultures of MTLn2(T42D) and MTLn3(T17D) grown in medium supplemented with 1% BSA, 123.0 and 351.7 ng of CSM protein were required, respectively (Table 1). The $\alpha$-Chy inhibitory activity present in the CSM/BSA preparations was not due to contaminating proteinase inhibitors that might have been carried over from BSA, since doses as high as 1.0 mg of BSA alone failed to give any detectable inhibition of enzyme activity (Table 1) in our assay. However, only 2.5 ng of FBS protein was sufficient to cause 50% inhibition of $\alpha$-Chy activity (Fig. 2; Table 1). Based on the data presented in Table 1, the CSM/FBS prepared from cultures of MTLn2(T42D) contains approximately 4.7-fold more enzyme inhibitory activity than the CSM/FBS derived from MTLn3(T17D), i.e., 4.50 versus 0.95 $I_{50}$ units/1 x $10^7$ cells and corresponds to nearly 10% of the activity detected in

![Inhibitory Concentration Response Curves of $\alpha$-Chy towards Various Conditioned Media](image)

**RESULTS**

**In Vitro Growth Rates of MTLn2(T42D) and MTLn3(T17D).** Fig. 1 depicts the growth characteristics of MTLn2(T42D) (Fig. 1a) and MTLn3(T17D) (Fig. 1b) tumor cells cultured in either RPMI/FBS or RPMI/BSA. MTLn2(T42D) cultures showed a population doubling time of $12$ h in RPMI/FBS versus $16$ h in RPMI/BSA, and both conditions resulted in similar final cell densities after $96$–$120$ h of growth (i.e., RPMI/BSA, $1.2 \times 10^3$ cells/cm$^2$; RPMI/FBS, $1.0 \times 10^3$ cells/cm$^2$). MTLn3(T17D) tumor cells, whether grown in RPMI/BSA or RPMI/FBS, exhibited a population doubling time of $15$ h...
supernatants were collected, concentrated, and assayed for the presence of proteinase inhibitory activity by using α-Chy as described in “Materials and Methods.”

Tumor cells were grown for 96 h in RPMI 1640 containing 1.0% BSA, washed 3 times with 15 ml of warm PBS and 10 ml RPMI 1640 containing either 1.0% BSA or 10% FBS were added to each culture. Cultures were returned to the incubator and after 20–24 h the media were removed, the cultures were washed 3 times with 15 ml of warm PBS, refed with 6 ml of RPMI 1640 devoid of any protein supplementation, and incubated for an additional 20–24 h. The culture supernatants were collected, concentrated, and assayed for the presence of proteinase inhibitory activity by using α-Chy as described in “Materials and Methods.”

FBS. In contrast, the amount of α-Chy inhibition produced by the CSM/BSA from cultures of MTLn2(T42D) and MTLn3(T17D) was 0.80 and 0.60 $I_0$ units/1 × 10$^7$ cells, respectively. This represents a decline in proteinase inhibitory activity of 82.2% for MTLn2(T42D) and 36.8% for MTLn3(T17D) when cultured in the absence of FBS. The loss of inhibitory activity was not a result of cell death since both tumor cell variants grow very well in medium supplemented with BSA and also remain healthy for at least 48 h when transferred to medium devoid of exogenous protein. These results strongly suggest that MTLn2(T42D) tumor cells when compared to their MTLn3(T17D) counterparts exhibit an increased capacity to retain and subsequently release active proteinase inhibitors capable of inactivating α-Chy. Moreover, the detection of inhibitory activity in CSM/BSA indicates that both metastatic variants possess the ability to synthesize their own active proteinase inhibitors.

Table 1 Relative amounts of proteinase inhibitory activity released by MTLn2(T42D) and MTLn3(T17D) metastatic variants

<table>
<thead>
<tr>
<th>Sample</th>
<th>Cultured in$^a$</th>
<th>$I_0$ (µg protein)</th>
<th>$I_0$ (units/mg protein)</th>
<th>$I_0$ (units/1 × 10$^7$ cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTLn2(T42D)</td>
<td>10.0% FBS</td>
<td>24.50</td>
<td>40.81</td>
<td>4.50</td>
</tr>
<tr>
<td>MTLn2(T42D)</td>
<td>1.0% BSA</td>
<td>123.00</td>
<td>8.13</td>
<td>0.80</td>
</tr>
<tr>
<td>MTLn2(T42D)</td>
<td>10.0% FBS</td>
<td>117.00</td>
<td>8.54</td>
<td>0.95</td>
</tr>
<tr>
<td>MTLn3(T17D)</td>
<td>1.0% BSA</td>
<td>351.70</td>
<td>2.84</td>
<td>0.60</td>
</tr>
<tr>
<td>BSA</td>
<td>&gt;1000.00</td>
<td>2.50</td>
<td>400.00</td>
<td></td>
</tr>
<tr>
<td>α-Chy</td>
<td></td>
<td>0.18</td>
<td>5555.50</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Tumor cells were grown for 96 h in RPMI 1640 containing 1.0% BSA, washed 3 times with 15 ml of warm PBS and 10 ml RPMI 1640 containing either 1.0% BSA or 10% FBS were added to each culture. Cultures were returned to the incubator and after 20–24 h the media were removed, the cultures were washed 3 times with 15 ml of warm PBS, refed with 6 ml of RPMI 1640 devoid of any protein supplementation, and incubated for an additional 20–24 h. The culture supernatants were collected, concentrated, and assayed for the presence of proteinase inhibitory activity by using α-Chy as described in “Materials and Methods.”

Analysis of Enzyme-Inhibitor(s) Complexes Formed by CSM/FBS and CSM/BSA Prepared from Cultures of MTLn2(T42D) and MTLn3(T17D) with [α-125I]Chymotrypsin. Nonreducing SDS-PAGE followed by autoradiography was used to detect specific complex formation between [α-125I]Chy and inhibitors present in the CSM. α-Chy-PI was used as a control to confirm the formation and detection of irreversible E-I complexes by our methods. Results obtained (Fig. 3) show that the addition of [α-125I]Chy to CSM/FBS and CSM/BSA from both metastatic variants results in the formation of heat- and SDS-stable E-I complexes as revealed by migration retardation of [α-125I]Chy. A major E-I complex with an apparent molecular weight of 79,000 was formed with CSM/FBS from both cell lines and comigrated with a similar complex formed with FBS and α-Chy-PI. Densitometric scanning of the autoradiogram (not shown) showed that the amount of the $M_r$ 79,000 complex resulting from the CSM/FBS of MTLn2(T42D) with [α-125I]Chy was approximately equal to that formed with FBS or α-Chy-PI, and 5-fold greater than that which was formed with CSM/FBS derived from the highly metastatic MTLn3(T17D) variant. Additional E-I complexes with apparent molecular weights in the range of 135,000 and 170,000 were also present in the CSM/FBS from MTLn2(T42D) and FBS. The $M_r$ 135,000 complex was also present in the CSM/FBS of MTLn3(T17D); however, it represented only 5.6% and 18% of that detected in FBS and CSM/FBS of MTLn2(T42D) cultures, respectively. The CSM derived from cells cultured in the presence of BSA either lacked [i.e., MTLn3(T17D)] or displayed barely visible amounts of the $M_r$ 79,000 complex [i.e., MTLn2(T42D)] and all higher molecular weight forms were absent. However, E-I complexes with apparent molecular weights of 50,000 and 44,000 were observed in the CSM/BSA of MTLn2(T42D) and MTLn3(T17D), respectively. A similar comigrating $M_r$ 50,000 E-I complex as that which was formed with the CSM/BSA of MTLn2(T42D) was also formed with FBS; but, no similar complexes were detected when [α-125I]Chy was incubated with 40 µg of BSA (Fig. 3, Lane 7). Therefore, it appears that these metastatic variants are capable of producing their own proteinase inhibitors; however, the major difference lies in their ability to retain and subsequently release active proteinase inhibitors derived from serum. Overall, the appearance of E-I complexes and their relative amounts are consistent with the results obtained in the kinetic assays.

Correlation of Tumor Cell-released Proteinase Inhibitory Activity In Vitro to Metastatic Potential In Vivo. In vivo results of the selected variants for their ability to colonize and grow in the lungs of syngeneic rats after i.v. injection of tumor cells, as well as their capacity to spontaneously metastasize to secondary sites from s.c. injections, is summarized in Table 2. The results indicate that MTLn2(T42D) tumor cells, whether injected directly into the lateral tail vein of rats or s.c. into the mammary fat pad region, were relatively inefficient in producing pulmonary or lymph node metastases as compared to highly metastatic MTLn3(T17D) tumor cells. Although 9 of 10 animals which had received MTLn2(T42D) tumor cells i.v. exhibited lung metastases, the median number of surface tumor lesions per lung was only 4 (range, 0–8). Lung tumor lesions were not observed in animals that had received MTLn2(T42D) cells s.c. in the mammary fat pad region; but, 3 of 9 animals did exhibit
from the spontaneous metastasis assay were prepared for pathological examination in order to verify the presence of tumor cells. Lymph nodes (i.e., lumbar, inguinal, and axillary nodes) were examined for the presence of metastases, using a dissecting microscope. Lung and lymph node tissues from the spontaneous metastasis assay were prepared for pathological examination in order to verify the presence of tumor cells.

Table 2 In vivo metastatic potential of MTLn2(T42D) and MTLn3(T17D) selected variants

<table>
<thead>
<tr>
<th>Metastatic variants</th>
<th>Route of inoculation</th>
<th>Size of s.c. tumors av. diameter mm ± s.d.</th>
<th>No. of lung surface tumor lesions/animal</th>
<th>No. of animals with lymph node metastasis</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTLn2(T42D)</td>
<td>i.v.</td>
<td>0, 1, 2, 3, 5, 6, 8, 8</td>
<td>8, 17, 19, 21, 25, 28, 32, 32, &gt;250*</td>
<td>0/10</td>
</tr>
<tr>
<td>MTLn3(T17D)</td>
<td>i.v.</td>
<td>32.24 ± 5.0</td>
<td>0, 0, 0, 0, 0, 0, 0, 0</td>
<td>3/9</td>
</tr>
<tr>
<td>MTLn2(T42D)</td>
<td>s.c.</td>
<td>20.33 ± 2.6</td>
<td>0, 0, 0, 8, 25, 25, 115, 175, &gt;250*</td>
<td>9/10</td>
</tr>
<tr>
<td>MTLn3(T17D)</td>
<td>s.c.</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* P = 0.01 (by Wilcoxon rank test) versus number of lung surface tumor lesions produced by i.v. injections of MTLn2(T42D) tumor cells.

** P < 0.001 (by Wilcoxon rank sum test) versus number of lung surface tumor lesions produced by i.v. injections of MTLn2(T42D) tumor cells.

Viable tumor cells (3 x 10^4 and 1 x 10^4) in 0.5 ml of RPMI 1640 were injected either i.v. into the lateral tail veins (experimental metastasis assay) or s.c. into the mammary fat pads (spontaneous metastasis assay) of 7- to 9-week-old F344 female rats, respectively. After 23 days (experimental metastasis assay) or 28 days (spontaneous metastasis assay) following tumor cell injections, the animals were killed by using metofane anesthesia, their s.c. tumors were measured, and lungs and lymph nodes (i.e., lumbar, inguinal, and axillary nodes) were examined for the presence of metastases, using a dissecting microscope. Lung and lymph node tissues were sectioned at 5-8 microns, and sections were stained with Hematoxylin and Eosin for histological examination. Tumor cell-mediated lesions were counted and scored, and the extent of lymph node involvement (i.e., axillary and lumbar nodes). In contrast, MTLn3(T17D) tumor cells caused lung metastases in 10 of 10 animals with a median of 25 (range, 8 to >250) lesions/lung when injected i.v., and 7 of 10 animals showed a median of 25 (range, 0 to >250) lung metastases when tumor cells were injected s.c. Histopathological evaluation of lungs revealed multiple foci of neoplastic cells within pulmonary capillaries (Fig. 4). Ninety % of the animals also displayed extensive inguinal, axillary, and lumbar lymph node involvement. The lymph nodes were often enlarged 2-5 times normal size and histological evaluation demonstrated nearly complete obliteration of lymph nodal architecture by sheets and nests of tumor cells (Fig. 5). Thus, the increased in vivo metastatic behavior of MTLn3(T17D) tumor cells appears to correlate with their decreased capacity to release, in vitro, active proteinase inhibitors either derived from serum or produced by the cells themselves.

**DISCUSSION**

The results presented in this report show that the spent culture medium from two isogenous rat mammary adenocarcinoma variants, selected for serum-independent growth in vitro, contain FBS-associated and tumor cell-produced proteinase inhibitors capable of inhibiting α-Chy activity. The CSM/FBS prepared from cultures of MTLn2(T42D) exhibit almost 5-fold greater inhibitory activity than that measured in the CSM/FBS of MTLn3(T17D). Analysis of SDS-PAGE and autoradiography revealed that the major inhibitor present in the CSM/FBS of MTLn2(T42D) and MTLn3(T17D) forms a heat- and SDS-stable E-I complex with an apparent molecular weight of 79,000, identical to that formed when FBS or α1-PI is incubated with [α-125I]Chy. These results suggest that this inhibitor might be identical or similar to serum α1-PI; however, its true identity remains to be determined. Although hepatocytes are known to be the principle source of α1-PI that circulates in plasma (28); other cell types, such as human rectal adenocarcinoma cells cultured in serum-free media, have been reported to produce α1-PI (29). Another member of the serpin family of proteinase inhibitors (reviewed in Refs. 30 and 31), α1-Achy, can also be synthesized by some tumor cells (32-34). α1-Achy, like α1-PI, is able to form a SDS-stable complex with α-Chy (35, 36); however, we were unable to detect E-I complexes with molecular weights suggestive of the presence of α1-PI or α1-Achy in the CSM/BSA of either MTLn2(T42D) or MTLn3(T17D) tumor cells. The absence of the formation of complexes with apparent molecular weights of >50,000 in the CSM of tumor cells that had not been exposed to FBS and the appearance of M, 79,000 to M, 170,000 complexes in the CSMs of cells exposed to FBS, that comigrated with those formed with FBS and a,-PI, suggests that these inhibitors originated from serum. We infer from these observations that the highly metastatic variants have a decreased capacity to retain and subsequently release active serum-derived proteinase inhibitors. The identity of the
inhibitors forming the E-I complexes with apparent molecular weights of 44,000 and 50,000 from the CSM/BSA of MTLn3(T17D) and MTLn2(T42D), respectively, has not yet been determined; their presence, nonetheless, indicates that these metastatic variants are capable of producing their own serine proteinase inhibitors. The observation that the $M_r$ 50,000 E-I complex is also present in the CSM/FBS of MTLn2(T42D) tumor cells and FBS alone, suggests that this tumor cell-produced inhibitor might be similar to an inhibitor found in serum. However, a computed molecular weight of approximately 27,000 for the free inhibitor (i.e., calculated by subtracting the apparent molecular weight of 23,000 for $\alpha_1$-Chy and assuming a 1:1 binding ratio of enzyme to inhibitor) does not lie within the ranges of molecular weights reported for any of the known serum proteinase inhibitors. It should be noted that exposure of tumor cell variants to FBS might have resulted in the induction of proteinase inhibitors by factors present in serum. Massot et al. (37) reported that among several human tumor cells studied, MCF7 tumor cells when exposed to 10 nM estradiol for 2.5 days, exhibited a 2-fold increase in the production of $\alpha$-Achy. Estradiol is present in FBS (mean, 0.7 nM, range, 0.6–1.0 nM; Ref. 38; mean, 0.04 nM, range, 4.0–75 pM⁴) and when FBS is used at 10% (a concentration typically used in cell culture media), the level of estradiol is in the range of 100- to 2500-fold less than that used by Massot et al. It, therefore, seems unlikely that the level of estradiol present in the FBS used in our experiments, as well as the relatively short time of exposure (i.e., 20–24 h), could result in the induction of synthesis and release of proteinase inhibitors detected in our experiments.

Fig. 5. Sections of lumbar nodes of rats given $1.0 \times 10^6$ MTLn3(T17D) tumor cells in the s.c. fat pad and necropsied 28 days later. a, entire node is markedly enlarged and lymphoid architecture is totally replaced by neoplastic cells. Area in box is enlarged in b. Ureter (U, arrow) is adjacent to capsule of involved node. Bar, 0.9 mm. b, enlargement of boxed area in a. Entire lymphoid architecture is replaced by nest of tumor cells (arrows) separated by reactive host stroma. Area of tumor cell necrosis (N, arrow) is compatible with ischemic necrosis of a rapidly growing tumor. Bar, 90 $\mu$m. c, enlargement of area from a. The metastatic carcinoma in lymph nodes was characterized by multiple nests of neoplastic cells separated by host-derived stromal tissue (S). The neoplastic cells have multiple or a single large intracytoplasmic vacuole and large vesicular nuclei. Bar, 30 $\mu$m. d, very little normal lymphoid tissue was present in lumbar lymph nodes at 28 days after tumor cell inoculation. This photomicrograph depicts cortical lymph node with complete infiltration of a germinal center (arrows) with neoplastic carcinoma cells. Paracortical lymphoid tissue is still partially intact but surrounded by sheets of neoplastic cells (arrow). Bar, 100 $\mu$m.

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⁴ Personal communication with Hazelton Biologics, Inc., and HyClone Laboratories, Inc.
Our results suggest that, in this model system for metastasis, tumor cells which exhibit a decreased ability to retain and release serine proteinase inhibitors, either derived from serum or produced by the tumor cells themselves, correlates to their increased metastatic behavior in vivo. Primary tumor invasion of surrounding normal tissue, with subsequent entry of tumor cells into vascular channels, is the first step in a series of complex tumor cell- and host-mediated events which can ultimately lead to the establishment of distant metastases (1). Once tumor cells have gained entry into blood or lymphatic vessels, extravasation can occur by invasion through vessel walls and entry of malignant cells into the organ parenchyma. In order for tumor cells to penetrate through these natural tissue barriers, they must be able to degrade interstitial matrix and basement membrane components such as collagens, proteoglycans, and laminin (7). The observation that malignant carcinomas in vivo lack a well defined basement membrane-like structure surrounding the tumor and often exhibit areas of tissue destruction adjacent to the invading tumor cells (7) is consistent with reports describing elevated levels of metalloproteinases, proteoglycanases, cysteine and serine proteinases (with chymotrypsin-like activity) secreted by invasive tumor cells in vivo (6).

The mechanisms involved in the regulation of the activities of proteinases during tumor invasion is at present poorly understood and is an important area of study. Since chymotrypsin-like enzymes are involved in tissue invasion, we decided to use α-Chy as the prototypic proteinase for the detection of serine proteinase inhibitors. Moreover, previous studies have shown that gp65, the major glycoprotein produced by several human breast cancer cell lines, is an inhibitor of α-Chy (32, 33).

Evidence that tissue- and/or tumor cell-derived proteinase inhibitors might play a role in regulating the action of tissue-degrading enzymes during tumor invasion has been presented by several investigators. For example, in a murine fibrosarcoma model, the production of TIMP, also known as collagenase inhibitor (reviewed in Ref. 39), by tumor cells in culture has been inversely correlated to their invasive potential (8). Halaka et al. (9), using explants of human meningiomas, have also made similar observations. Purified TIMP was found to inhibit the invasion of the human amniotic membrane by murine M5076 reticulum sarcoma cells in vitro (40). More recently, K烘ka et al. (10) were able to confer oncogenicity and metastatic capacity to Swiss 3T3 cells that were genetically engineered to synthesize complementary antisense TIMP RNA. Reich et al. (41) demonstrated that serine proteinase inhibitors and a synthetic collagenase inhibitor were able to independently block the invasion of basement membranes by metastatic cells in vitro. Leupeptin, which inhibits thrombin, elastase, kallikrein, cathepsin B, trypsin, and papain, when administered in vivo was found to markedly suppress the formation of irreversible complexes. The results presented here clearly show a strong inverse correlation between the presence of these proteinase inhibitors in vitro and metastatic behavior in vivo; and further studies to identify and fully characterize these inhibitors will be needed to establish their precise role in regulating tumor invasion.

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