Abstract. Two proteases cathepsin D (cath D) and urokinase plasminogen activator (uPA) are tissue markers associated with an increased risk of metastasis in breast cancer. We investigated whether cath D, the major aspartyl protease overexpressed by breast cancer cells can trigger a proteolytic cascade via activation of plasminogens at the extracellular pH measured in hypoxic tumors. The effects of the aspartyl protease inhibitor pepstatin on the plasminogen activator (PA) system were analysed by conditioning media of human MDA-MB231 breast cancer cells at pH 6.6 and pH 7.4. Zymography analysis of culture media showed that pepstatin inhibited the secreted activity of tissue-type plasminogen activator (tPA) but not that of uPA. tPA was identified on the basis of the molecular weight, the immunoreactivity with relevant antibodies and the resistance to amiloride, a specific uPA inhibitor. The secreted tPA activity measured by a chromogenic assay in the presence of amiloride was also inhibited by pepstatin at pH 6.6. Surprisingly, pepstatin did not affect secreted tPA protein concentration but markedly increased the amount of the secreted plasminogen activator inhibitor-1 (PAI-1). We conclude that cath D overexpressed by these cells, stimulates at pH 6.6, but not at neutral pH, the extracellular PA proteolytic activity indirectly via PAI-1 proteolysis. This suggests that cath D at acidic pH close to the hypoxic regions of solid tumors, contributes to trigger a proteolytic cascade facilitating cancer cell invasion and metastasis.

Introduction

High expression and over-secretion of cathepsin D (cath D) have been shown in several independent studies to be associated with an increased risk of recurrence and metastasis in breast cancer (1-3). Since the inactive 52 kDa procathepsin D is secreted by cancer cells both in culture under routine conditions at pH 7.4 (4,5) and in pleural effusion of breast cancer (6), its maturation in acidic compartment is required to be proteolytically active. In this investigation we raised two major questions. Are cancer cells, cultured at the extracellular pH of hypoxic solid tumors (7,8) able to stimulate the cath D activity, and in this case, would cath D be able to activate the plasminogen activator system since a high uPA concentration in breast cancer cytosol was associated with poor prognosis (9,10)?

We therefore monitored serum-free culture conditions at pH 6.6 approaching the acidity of tumor microenvironments allowing the survival of invasive MDA-MB231 breast cancer cells without cell lysis. We then tested the effects of medium acidification mediated by cath D activity on the secretion of uPA, tissue-type plasminogen activator (tPA) and PA inhibitor-1 (PAI-1). The two secreted plasminogen activators and their activities were analysed in the presence or absence of pepstatin, a specific aspartyl protease inhibitor.

Materials and methods

Cell culture and preparation of conditioned media. MDA-MB231 breast cancer cells were cultured in duplicate 6-well plates in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum to 80% confluence. To test the effect of an acidic pH on lysosome distribution and cath D routing, cells were transferred on glass coverslips and medium was changed to a 50 mM HEPES-buffered Ringer's solution with 70 mM Na acetate as described (11). To prepare conditioned media during longer time of treatment, the cells were transferred in duplicate in 6-well plates in DMEM solution as described (12). Briefly, the culture medium was DMEM without FCS, with Hank's balanced salt solution. It was adjusted to pH 6.6.
or 7.4 using HCO$_3$/HCl according to the equation [HCO$_3$] = (1.52 mM of CO$_2$) x [10 (pH 6.24)]. In experiments with peptatin A (50 or 100 μM), this aspartyl protease inhibitor was dissolved in DMSO and its effect was compared to control media containing the same amount of DMSO alone. The pH of conditioned media measured before addition to the cells and at the end of incubation were controlled to be constant. Under these conditions, MDA-MB231 cells were viable for at least 4 days. Cell number was evaluated by DNA assay using the DABA colorimetric method (13).

Media conditioned by these cells during increasing periods of time were normalised to the number of cells (determined by DNA assay), and centrifuged to remove cell debris. The supernatant was then layered on a PAGE for zymography or western blot analysis or were assayed for tPA activity and for tPA and PAI-1 antigen concentrations.

Analysis of plasminogen activators (PA) activity by casein-plasminogen zymography. PA activity was analysed after separation by electrophoresis in 10% polyacrylamide SDS gel copolymerized with 1 mg/ml of casein (Sigma, St. Louis, MO, USA) and 30 μg/ml human plasminogen (Sigma) under non-reducing conditions (without mercaptoethanol) and in the absence of serine protease inhibitors, as described (14,15). The caseinolytic bands were revealed and quantified by scanning. Molecular weight markers (Amersham, Piscataway, NJ, USA) were run in parallel.

Plasminogen activator enzymatic assay. PA activity in conditioned media was determined in triplicate using the human PA chromogenic enzymatic kit (Assay pro ref CT1001, AssaySense, St. Charles, MO, USA) according to instructions of this laboratory. This assay was not specific for tPA or uPA. Briefly, plasminogen activator enzymatic assay.

The 3-day conditioned media of MDA-MB231 cells, collected after 1 day of incubation, the reaction was stopped by freezing. Samples were then boiled in electrophoresis buffer for 3 min, and fragments of PAI-1 were separated on a 12% polyacrylamide SDS-PAGE. The SDS-PAGE was stained with Brilliant Blue.

Immunolocalization of cath D and validity of the cath D antibodies. MDA-MB231 cells were cultured on glass coverslips at pH 7.4 or 6.6 for 3 h. They were then fixed with paraformaldehyde and glutaraldehyde and permeabilized by Triton X-100. Lysosomes were stained using the anti-cath D M1G8 mAb (18), the cath D swine polyclonal antibodies (kindly provided by Dr M. Fusek) or the anti-LAMP1 antibody (Abcam). The M1G8 mAb used (Figs. 1 and 2) recognises all forms of cath D, while the M2E8 mAb is specific of the pro-enzyme. These antibodies have been characterized previously (18,19) and the references within).

Results

Effect of the acidic pH on MDA-MB231 cells, lysosome localization and cath D level. MDA-MB231 breast cancer cells were plated in 12-well plates to reach 30% confluence and then incubated in the FCS free media at pH 7.4 or 6.6 for up to 3 days. Their growth was decreased by 35% at pH 6.6 compared to pH 7.4, but not altered by pepstatin A, at either pH value (results not shown). We have also verified that cells plated at 80% confluence, remained quiescent without cell lysis at pH 6.6 for at least 36 h allowing the preparation of conditioned media for secreted proteins analysis (data not shown).

As shown on Fig. 1, the lysosomes stained by different cath D antibodies or Lamp1 antibodies, were delocalised at the cell periphery by medium acidification from pH 7.4 (30% at the cell periphery) to pH 6.6 (70% at periphery). The effect was progressive from pH 7.4 to 6.3 and was optimal at pH 6.6 which was chosen for further experiments. This peripheral location of lysosomes at pH 6.6 was rapid, within 7 min and stable for 3 days (Fig. 2). It was rapidly reversible at pH 7.4 indicating that the cells were still viable (data not shown).

Zymography analysis of the cath D stimulation of the proteolytic activity of secreted plasminogen activators. The conditioned media of MDA-MB231 cells, collected after 1
to 3 days of culture at pH 7.4 or 6.6, were analysed for PA activities on a non-reducing SDS-PAGE containing plasminogen and casein as described in Materials and methods. In acidic and neutral media the same caseinolytic large bands were observed migrating with apparent molecular weight of 110-130, 63-75 and 45 kDa. However, when cells had been treated with pepstatin, the caseinolytic bands of higher molecular weight (63-75 kDa) were decreased in acidic conditions but 45 kDa bands were unaffected (Fig. 3). The molecular weights of the cath D stimulated bands were in agreement with those of the unbound tPA (63-75 kDa) and of a PA/PAI-1 complex (110-130 kDa) (16,20,21). The absence of caseinolytic bands in PANE analysis performed in the same experiments without plasminogen (Fig. 3A, lanes 9 and 10) or without conditioned media showed an effect due to the activation of plasminogen rather than the activation of other proteases secreted or present in the gel. The 45 kDa band (Fig. 3) corresponded to uPA on the basis of its molecular weight and specific sensitivity to amiloride (16,22) as shown in Fig. 4. This band was not modified by pepstatin treatment suggesting that uPA activity was not stimulated by cath D activity (Figs. 3 and 4). Quantification by scanning of lanes 1 to 4 of the zymograph (Fig. 3A) showed that after 3 days of conditioning, the activity of the different molecular weight PA forms, was decreased at the acidic pH (Fig. 3B). This decreased PA-induced caseinolytic activity at pH 6.6 compared to pH 7.4 was reproducibly observed in independent experiments (Fig. 5) and was associated with a decrease of the secreted cath D (see previous section). Fig. 3B also shows that pepstatin decreased specifically the 75 to 63 kDa activities at pH 6.6 but not at pH 7.4 while the uPA activities were not altered. The effect of pepstatin on the 63-75 kDa lytic bands was slow and optimal after 3 days of treatment (Fig. 5).

**Effect of pepstatin at pH 6.6 on the activity and protein level of tPA and PAI-1 in the conditioned media.** The effect of pepstatin on the PA proteolytic activity of conditioned media conditions but 45 kDa bands were unaffected (Fig. 3). The molecular weights of the cath D stimulated bands were in agreement with those of the unbound tPA (63-75 kDa) and of a PA/PAI-1 complex (110-130 kDa) (16,20,21). The absence of caseinolytic bands in PANE analysis performed in the same experiments without plasminogen (Fig. 3A, lanes 9 and 10) or without conditioned media showed an effect due to the activation of plasminogen rather than the activation of other proteases secreted or present in the gel. The 45 kDa band (Fig. 3) corresponded to uPA on the basis of its molecular weight and specific sensitivity to amiloride (16,22) as shown in Fig. 4. This band was not modified by pepstatin treatment suggesting that uPA activity was not stimulated by cath D activity (Figs. 3 and 4). Quantification by scanning of lanes 1 to 4 of the zymograph (Fig. 3A) showed that after 3 days of conditioning, the activity of the different molecular weight PA forms, was decreased at the acidic pH (Fig. 3B). This decreased PA-induced caseinolytic activity at pH 6.6 compared to pH 7.4 was reproducibly observed in independent experiments (Fig. 5) and was associated with a decrease of the secreted cath D (see previous section). Fig. 3B also shows that pepstatin decreased specifically the 75 to 63 kDa activities at pH 6.6 but not at pH 7.4 while the uPA activities were not altered. The effect of pepstatin on the 63-75 kDa lytic bands was slow and optimal after 3 days of treatment (Fig. 5).
was also evaluated by a PA colorimetric assay. After 3 days of culture at pH 6.6, the total PA activity and the amiloride resistant tPA activity were partly inhibited by pepstatin (Table I, compare lanes a and b).

In order to confirm that the effect of cathepsin D was not due to an increase of the secreted tPA, we estimated its concentration using western immunoblot analysis. As shown in Fig. 6A, the human tPA present as a large 75 kDa band appeared unaffected in the presence of pepstatin at pH 6.6. Excluding a non-specific 55 kDa band also revealed with the secondary antibodies alone (lanes 4-6), the tPA antibodies recognised at pH 7.4 a 75 kDa band corresponding to tPA and a predominant 110 kDa band corresponding to tPA/PAI-1 inhibitor complexes (lane 1) as previously described (20,21). At pH 6.6 the amount of the secreted tPA was decreased compared to pH 7.4, but pepstatin had no effect on the level of the 75 kDa band (Fig. 6). While the 110 kDa entity was predominant at pH 7.4, (lane 1) it was not seen at pH 6.6 (lanes 2 and 3) which is consistent with a dissociation of the tPA/PAI-1 inhibitor complex facilitated.
at an acidic pH as shown previously (14,15,25). The contrast between the larger lytic bands (75 to 63 kDa) of the zymography experiments, compared to the distinct 75 kDa bands of the western blot analysis, might be due to the absence of added serine protease inhibitors and mercaptoethanol in the zymography experiments.

These data suggest that the mechanism of the cath D activation of tPA was probably indirect and due to the degradation of a tPA inhibitor by cath D. We demonstrate that pepstatin markedly increased PAI-1 concentration in the same conditioned media (Table I, line 4). PAI-1 degradation by cath D has been previously described in monocytes at a more acidic condition.

Table I. Effect of pepstatin on PA activities, tPA and PAI-1 concentrations secreted by MDA-MB-231 cells at pH 6.6.

<table>
<thead>
<tr>
<th></th>
<th>Cell medium after 72 h culture at pH 6.6</th>
<th>Cell medium after 72 h culture at pH 6.6 + 10⁻⁴ M pepstatin</th>
<th>Student’s t-test: p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) PA activity % control ± SD</td>
<td>100.0±15.3 (2)</td>
<td>60.0±7.3⁺ (2)</td>
<td>0.06</td>
</tr>
<tr>
<td>2) tPA activity (amiloride resistant) % control ± SD</td>
<td>100.0±29.5 (6)</td>
<td>68.0±4.7⁺ (6)</td>
<td>0.048</td>
</tr>
<tr>
<td>3) tPA level in ng/mg protein</td>
<td>9.86±0.73 (3)</td>
<td>11.02±0.52⁺ (3)</td>
<td>0.039</td>
</tr>
<tr>
<td>4) PAI-1 level in ng/mg protein</td>
<td>269.3±10.9 (3)</td>
<td>405±17.0⁺ (3)</td>
<td>0.00001</td>
</tr>
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</table>

MDA-MB-231 cells were incubated in triplicates for 72 h in serum-free medium at pH 6.6 with or without 10⁻⁴ M pepstatin. Conditioned media were prepared as in Materials and methods for zymography, they were not concentrated but centrifuged to remove eventual detached cells. They were assayed for total PA activities by the PA chromogenic activity Zymutest assay, 1) without amiloride or 2) for tPA activities in the presence of 1 mM amiloride. The amiloride resistant PA corresponded to about 30% of the total PA activity. Results are in % of the activity without pepstatin. 3) tPA antigen concentrations were assayed by the Imubind (PA ELISA kit). 4) PAI-1 concentrations were assayed by the ELISA Femtelle kit and expressed per mg of total secreted protein. Values were obtained from 3 independent experiments and triplicate assays for each experiment. ⁺The pepstatin effect is at the limit of significance. ⁺⁺A highly significant pepstatin effect.
(23). We complete these data in Fig. 7 by finding that cath D slowly degraded pure PAI-1 even at pH 6.0 in a cell free system. Collectively, these data indicate that cath D increased the activity of secreted plasminogen activators (PA) at acidic pH and decreased in the same conditions the level of PAI-1.

Discussion

This study aimed to specify whether the weak acidity (pH 6.5 to 6.7) of the extracellular milieu of hypoxic solid tumors (7,8) allows the activation of pro-cath D and alters the secreted activities of plasminogen activators.

We show that under a weakly acidic condition, MDA-MB231 cells were able to survive and to secrete a proteolytically active cath D. Therefore, in addition to its mitogenic activity as a ligand at a neutral pH (24), cath D can also be activated as a protease when breast cancer cells are exposed to an extracellular pH of 6.6. This mild acidic pH can be observed in vivo in aggressively solid tumors (12,25).

At pH 6.6, cath D stimulated the liberation in the culture medium of an active plasminogen activator resistant to amiloride and corresponding most likely to tPA. The MDA-MB231 cells are more aggressive and more efficient than MCF7 cells in spontaneously acidifying an extracellular milieu (12). They overexpress constitutively cath D (5) corresponding to a basal-type breast cancer. By contrast the estrogen receptor positive luminal type MCF7 cells require estradiol to express and secrete both pro-cath D (5) and tPA (26). We show here that the MDA-MB231 cells secrete both tPA, uPA and PAI1 and that cath D can increase PA activities via its proteolytic activity inhibited by pepstatin.

The monolayer cell culture on plastic, used in our study, is far from the in vivo condition. However, the cath D effect on PA activity might even be more important in vivo and within the microenvironment of a solid tumor. Actually both tPA activity and tumor aggressiveness were markedly increased in MDA-MB231 cells after their in vivo passage as orthotopic xenograft tumors in mice, indicating that the rodent microenvironment including stromal and endothelial cells, cooperated to increase tPA activity (27).

We show here additional evidence that cath D can be activated in vivo to behave as a protease. Previous reports showed that cath D in MCF7 cells stimulated FGF2 cellular uptake from an embedded extracellular matrix (28). Conversely, the addition of a KDEL retention signal for endoplasmic reticulum to pre-procath D, inhibited in vivo both cath D maturation and experimental metastasis in mice (29) underlining the requirement of a proteolytic activity to stimulate metastasis.

The mechanisms of the presence of secreted active forms of cath D and of its specific effect on PA activities are not fully understood. Active secreted cath D could be due to the auto-activation of procath D by removal of its pro-fragment (30) or to the rapid displacement of lysosomes at the cell periphery facilitating the secretion of lysosomal active cath D (Figs. 1 and 2). Whether this rapid lysosome delocalisation explains the secretion of mature cath D in the medium as proposed for cath B secretion (31) was not investigated in the present study.

The mechanism of the increased activity of the secreted tPA by cath D is not due to an increased amount of tPA, as shown by western blot analysis and immunoassay of the antigen. Since the zymogen and the two-chains forms of tPA display similar activity (32,33), the most likely explanation is that the effect of cath D is indirect. In fact the secreted PAI-1 is in large excess compared to PA in several cell lines (34) and modulation of tPA activity by PAI-1 is central in endothelial cells (35). Moreover, PAI-1 is a specific substrate of cath D, but not of cysteinyl cathepsins, as shown in human monocytes (23). The fact that we show here at pH 6.6 a significant increase of PAI-1 with pepstatin and the proteolysis of recombinant PAI-1 by cath D supports an indirect stimulation of the secreted PA activity via PAI-1 proteolysis. It is however intriguing that the degradation of PAI-1 by cath D stimulates specifically tPA activity measured by zymography while cath D appears to stimulate both uPA and tPA activities (compare lines 1 and 2 of Table I) when measured in the conditioned media before separation of the two enzymes by electrophoresis (Table I). This might be due to a different affinity of PAI-1 for tPA and for uPA (36). However, we cannot exclude other mechanisms at the cell surface where several receptors bind these proteases, such as the Annexin II/plasminogen receptor (37) and the LRP receptor which can bind both tPA/PAI-1 complex (38) and cath D (39).

The cath D proteolytic activity facilitating indirectly PA activation completes the scheme of a very complex proteolytic network leading to the stimulation of invasion, angiogenesis and tumor growth (40). Procath D can be autoactivated in vitro at an acidic pH (4,5,30) to trigger a proteolytic cascade when liberated with other proteases in the extracellular milieu of tumors. Activation of procathepsin B (41) and procathepsin L (42) by cath D had been shown in vitro but at the more acidic pH found in lysosomes, thus limiting its biological significance. This study introduces tPA as an additional partner in this complex proteolytic cascade to modulate invasion, metastasis and angiogenesis.

The significance and role of tPA and PAI-1 in cancer is debated (43). On one hand, several experimental studies indicate that tPA facilitates tumor progression and several mechanisms have been proposed (27,39,44,45). Gene invalidation in mouse showed that tPA cooperates with uPA in stimulating cell survival (46). However, clinical studies have underlined tPA level as a marker of good prognosis in breast cancer (47,48) in contrast to uPA (9). The reason for these discrepancies is not currently understood. Moreover, PAI-1 by inhibiting tPA activity has also been proposed to decrease the stress-induced senescence activity of the wild-type p53 (49). Thus cath D, by degrading PAI-1 and IGF-BP3 (50), might also interfere with the PAI-1/IGF-BP3 cascade (51) to stimulate cancer cells.

To conclude, we show here that cath D in breast cancer cells at pH 6.6, but not at neutral pH, stimulates as a protease the activity of secreted plasminogen activators probably by degrading PAI-1. This effect might also take place in vivo in poorly vascularised regions of solid tumors to modulate invasion and tumor growth.

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References


