Decreased secretion of Cathepsin D in breast cancer *in vivo* by tamoxifen: mediated by the mannose-6-phosphate/IGF-II receptor?

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**Summary**

The lysosomal protease Cathepsin D (Cath D) is associated with increased invasiveness and metastasis in breast cancer. Both estrogen and tamoxifen have been reported to increase Cath D, which seems to contradict the efficacy of tamoxifen as an adjuvant for estrogen dependent breast cancer. Cath D is bioactive in the extracellular space but very little is known about hormonal regulation of secreted Cath D *in vivo*. In this study we used microdialysis to sample the extracellular fluid in estrogen receptor positive MCF-7 tumors in nude mice. We show that tamoxifen in combination with estradiol decreased secreted Cath D compared with estradiol treatment only in solid tumors *in situ*. Cell culture of MCF-7 cells revealed that estradiol and tamoxifen increased intracellular proteolytic activity of Cath D in a similar fashion whereas secretion of Cath D was increased by estradiol and inhibited by tamoxifen. Immunofluorescence showed that estradiol located Cath D to the cell surface, while tamoxifen accumulated Cath D to dense lysosomes in perinuclear regions. Moreover, tamoxifen increased the intracellular transporter of Cath D, the mannose 6-phosphate/IGF-II receptor (M6P/IGF2R). In contrast, estradiol decreased the levels of this receptor. Thus, secretion of Cath D is hormone dependent and may be mediated by altered expression of the M6P/IGF2R.

Our results highlight the importance of measurements of proteins in all compartments where they are biological active and show that microdialysis is a viable technique for sampling of Cath D *in vivo*.

**Introduction**

Breast cancer accounts for almost 30% of all cancers among women in the Western world today and half of these women will die from metastatic disease [1]. In order to spread, tumor cells overproduce proteases, which permit cancer cell invasion by digestion of the extracellular matrix of the surrounding tissue [2]. Cathepsin D (Cath D), a lysosomal aspartic protease, has been suggested to be a prognostic indicator for recurrence and/or metastasis in breast cancer [3–5]. However, this is still controversial primarily due to inconsistent findings using non-quantitative methods such as immunohistochemistry [6]. Previous studies have found that breast cancer cells produce abnormally high amounts of Cath D, which is secreted rather than stored in lysosomes [7]. This suggests that measuring secreted Cath D *in vivo* in the extracellular space of tumors would be more relevant for predicting the invasive potential of tumors. Cath D is synthesized as a 52-kDa precursor protein, which is recognized by the mannose 6-phosphate/insulinlike growth factor-II receptor (M6P/IGF2R) in Golgi and transported via prelysosomal vesicles or late endosomes to lysosomes [8]. In breast cancer cells this receptor appears to be down regulated or poorly functional leading to increased secretion of Cath D [6]. At acidic pH, Cath D is released from the M6P/IGF2R and cleaved to an intermediate 48 kDa form which is further processed to the mature form consisting of two polypeptides of 30 and 12 kDa, respectively [9]. The processed secreted forms have been suggested to be the most important for metastatic spread in breast cancer [10–13].

Estrogen exposure is considered a major risk factor for development of breast cancer and the majority of breast cancers have maintained their hormonal dependency [14, 15]. Therefore, strategies aiming at
reducing the influence of estrogen on breast cancer cells have been developed. The most frequently used endocrine therapy in all stages of breast cancer is tamoxifen. Tamoxifen is a partial agonist to the estrogen receptor and has positive as well as negative effects on other organ systems. In estrogen receptor positive (ER+) cells, overexpression of Cath D is induced by estrogen [5, 16]. Tamoxifen has also been reported to increase intracellular levels of Cath D and affect the secretion of Cath D [16–20]. However, this would seem to contradict the efficacy of tamoxifen as an adjuvant for estrogen dependent breast cancer.

In the present study we investigate effects of estrogen and tamoxifen on the secretion of Cath D and the regulation of M6P/IGFIIIR in breast cancer in vitro and in vivo. Moreover, we introduce microdialysis as a technique for detecting extracellular secreted Cath D in solid tumors.

Materials and methods

Cells and culture conditions

A human breast adenocarcinoma cell-line MCF-7 (ER+ and PR+; ATCC, Manassas, VA, USA) was used. Cells were cultured in Dulbecco’s modified Eagle’s medium without phenol red supplemented with 2 mM glutamine, 50 IU/ml penicillin-G, 50 μg/ml streptomycin and 10% fetal bovine serum at 37°C in a humidified atmosphere containing 5% CO2. Cell culture media and additions were from GIBCO, Paisley, UK if nothing else is stated. Prior to experiments, cells were trypsinized (0.05% trypsin and 0.02% EDTA) and seeded into Petri dishes (Costar, Cambridge, MA, USA), 10,000 cells/cm2 dish. Cells were incubated for 1 day and then treated for 7 days with or without 10−8 M estrogen (17β-estradiol; Apoteket, Umeå, Sweden), 10−6 M tamoxifen (Sigma, St. Louis, MO, USA) or a combination of estradiol and tamoxifen. Hormones were added in serum free medium made of a 1:1 mixture of nutrient mixture F-12 (HAM) and Dulbecco’s modified Eagle’s medium without phenol red supplemented with 10 μg/ml transferrin (Sigma), 1 μg/ml insulin (Sigma) and 0.2 ng/ml bovine serum albumin (Sigma). The medium was changed every day.

Determination of Cath D activity

Cath D activity was determined as described by Barrett using hemoglobin as a substrate [21]. Culture medium was removed and cells were washed once in PBS (900 μM CaCl2, 490 μM MgCl2, 150 mM NaCl, 2.7 mM KCl, 1.5 mM KH2PO4 and 8.1 mM Na2HPO4; pH 7.5) and were then exposed to three cycles of freezing and thawing. Cells were collected in 1 ml sodium phosphate buffer (2 mM, pH 6.5) on ice and DNA content was determined in a 50-μl aliquot of the suspension, using Hoechst 33258 as described by Labarca and Paigen [22]. Cell-lysate (500 μl) was then mixed with 250 μl sodium formate buffer (1 M, pH 3.5) and 250 μl 4% hemoglobin (Sigma H-2500) and after incubation (60 min, 45°C), 5 ml 3% trichloric acid was added. The suspension was filtered and the amount of released peptide was analyzed using the method described by Lowry with slight modification [23]. For determination of Cath D secretion to the culture medium, medium from the last 24 h of incubation was collected and analyzed as described above. Cath D activity is given in arbitrary units per hour/μg DNA or % of the control values. To determine the percentage of protease activity that was Cath D specific in the lysate, 2.3 μM pepstatin A, a specific inhibitor of aspartic proteases, was added to selected samples [24].

Western blot analysis

Cells were lysed in 63 mM Tris–HCl (pH 6.8), 10% glycerol, 2% SDS, 5% β-mercaptoethanol and 0.05% bromphenol blue. For Cath D determination 40 μg protein was fractionated for each sample by 12% SDS-polyacrylamide gel. For analysis of the mannose-6-phosphate receptor, non-reducing conditions was used, that is, β-mercaptoethanol was omitted from the lysis buffer, and the samples (50 μg) were loaded on a 4–20% SDS-polyacrylamide gel and subjected to electrophoresis. After electro-transfer onto nitrocellulose membranes, the blots were blocked by TBS (50 mM Tris buffer supplemented with 0.15 M NaCl), 5% skimmed milk and 0.1% Tween-20 for 90 min at room temperature and washed in TBS. Thereafter the membranes were exposed for 16 h at 4°C to either a mouse anti human Cath D monoclonal antibody (1:1000, DAKO, Glostrup, Denmark) or a mouse anti-mannose-6-phosphate receptor antibody (1:1000; R & D Systems) followed by a horse radish peroxidase (HRP)-conjugated goat anti-mouse antibody (1:1000, DAKO). Bands were visualized using enhanced chemiluminescence (Amersham Pharmacia Biotech, Buckinghamshire, UK).