Induction of cathepsin D protein during estrogen carcinogenesis: possible role in estrogen-mediated kidney tubular cell damage

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We have proposed that an early step in estrogen carcinogenesis in the hamster kidney is tubular damage followed by reparative cell proliferation. This tubular injury is progressive and increases in severity with continued estrogen treatment; one pertinent feature is a marked rise in the number of both secondary and tertiary lysosomes. Data presented herein indicate that cathepsin D, an estrogen-responsive lysosomal proteolytic enzyme, is increased in the kidney following estrogen treatment in the hamster. Three isoforms of cathepsin D were detected in estrogen-treated kidneys, 52, 31, and 27 kDa, the major being 52 kDa. At 1 and 3 months of estrogen treatment, 52-kDa cathepsin D content increased 1.4- to 1.6-fold. These changes coincided with a rise in renal estrogen receptor levels during the same estrogen treatment periods. More pronounced rises in cathepsin D levels, 2.7- and 3.5-fold, were seen after 4 and 5 months of estrogen treatment, respectively. A concomitant, 3.0- to 4.0-fold rise in estrogen receptor content was also observed. At 5 months of estradiol or DES treatment, both 27- and 31-kDa isoforms were present in hamster kidneys, in addition to the 52-kDa form. Neither progesterone nor DHT treatment affected the untreated levels of cathepsin D. Interestingly, either concomitant tamoxifen or DHT and estrogen treatment prevented the rise in cathepsin D and estrogen receptor content observed after estrogen treatment alone. Primary estrogen-induced renal tumors and their metastases exhibited markedly elevated levels of all three isoforms of cathepsin D. Immunohistochemical analysis of cathepsin D in kidney sections confirmed the Western blot findings. These data suggest a novel role for estrogen-induced cathepsin D in the hamster kidney during tumorigenesis; that is, mediating renal tubular damage as a prelude to reparative cell proliferation, thus initiating a multi-step estrogen-driven process which leads to renal tumor formation.

Introduction

There is substantial evidence from in-vivo studies that an early step in estrogen carcinogenesis in the hamster kidney is tubular damage followed by reparative cell proliferation (1,2). Characteristic of renal tubular injury following chronic estrogen exposure of male hamsters are both losses of and abnormal microvilli, accumulation of lipid droplets, and a marked rise in the number of both secondary and tertiary lysosomes, none of which is seen in any of the renal tubules from untreated age-matched animals (2). The kidney damage is progressive and increases in severity, particularly after the third month of estrogen treatment. The mechanism of estrogen-mediated renal tubular damage in the hamster, detected by electron microscopy as early as 1 month after estrogen treatment, has until now remained elusive.

Numerous in-vitro studies have suggested the possibility that potential reactive intermediates, resulting from the metabolism of estrogens, may be the causative agents eliciting renal cell injury during chronic and prolonged exposure to estrogens (3–5). However, the relatively high estrogen concentrations (10–100 µM) required to generate significant quantities of such estrogen metabolites in in-vitro assays (6–9) are inconsistent with the very low in-vivo estrogen levels found in the kidney (0.004 ng/mg protein) and serum (2.36 ng/ml), a condition which yields essentially 100% renal tumor incidence (10).

Previous studies have extensively documented that the hamster kidney is a bona fide estrogen target tissue that possesses a functional estrogen receptor and inducible progesterone receptor as a result of estrogen treatment (11–13). Importantly, it has been shown that estrogens specifically induce renal tubular cell proliferation in culture grown under serum-free chemically-defined conditions at physiologic concentrations (1 nM) (14).

In estrogen receptor-positive breast cancer tissues, cathepsin D (EC 24.23.5), a lysosomal aspartyl endopeptidase and estrogen-responsive proteolytic enzyme, is frequently elevated, and is induced in hormone-dependent breast cancer cell lines (15,16). Moreover, it has been suggested that, in breast cancer cells, this secreted cathepsin D is involved in extracellular matrix degradation and the metastatic spread of malignancy (17,18). Additionally, it has been reported that both cathepsin D and B exhibit proteolytic activity on basement membranes, possibly causing them to rupture (17,19), and that the 52 kDa cathepsin D precursor promotes breast cancer cell growth of estrogen-depleted MCF-7 cells (19).

The data presented demonstrate that potent estrogens administered in vivo specifically elicit a marked rise in hamster kidney cathepsin D content, shown by immunohistochemical and Western blot analyses. Moreover, a concomitant rise in renal estrogen receptor content, during the same time intervals following estrogen treatment, coincides with the increased cathepsin D levels. These findings provide strong evidence that the rise in estrogen-induced cathepsin D may contribute

*Abbreviations: BSA, bovine serum albumin; DES, diethylstilbestrol; DHT, 5α-dihydrotestosterone; E1, estrone; E2, 17β-estradiol; EE, 17α-ethinylestradiol; ER, estrogen receptor; ERE, estrogen response element; Mt, metastasis; PBS, phosphate-buffered saline; PAP, peroxydase anti-peroxidase; PR, progesterone receptor; T, tumor; Tx, Tamoxifen.
Chemical Co., St Louis, MO) diluted 1:50 was then applied for 1 h, followed by 1 h incubation with rabbit PAP complex (Sigma Chemical Co., St Louis, MO) diluted 1:500. Sections were rinsed with phosphate buffered saline (PBS) pH 7.4 after each change of antibody. The signal was visualized by diaminobenzidine (DAB) and H2O2. The specificity of the signal was controlled by mounting two serial kidney sections in the same slide and using one section as a control. Control sections received 1% BSA instead of the cathepsin D antibody. Finally, the sections were counterstained with hematoxylin, rinsed, dehydrated, cleared, and mounted.

**Results**

**Cathepsin D and estrogen receptor levels during estrogen-induced renal tumorigenesis**

Cathepsin D and estrogen receptor content, assessed by Western blotting, were determined at various monthly intervals of DES treatment (Figure 1). Three isoforms of cathepsin D with kDa values of 52, 31, and 27 were observed, the major isoform being 52 kDa. After 1 and 3 months of estrogen treatment, small but consistent increases in 52 kDa cathepsin D content (1.4- to 1.6-fold) were observed, compared to age-matched untreated animals (Figure 1A). During the same estrogen treatment periods, similar increases in renal estrogen receptor levels (1.6- to 1.8-fold) were found (Figure 1B). More pronounced rises in 52 kDa cathepsin D levels (2.7- and 3.5-fold) were detected after 4 and 5 months of estrogen treatment, respectively. The 27 kDa cathepsin D isoform was present in kidneys from 3–5 months of DES treatment (Figure 1A). At 4 and 5 months of estrogen treatment, estrogen receptor content also rose 3.0- and 4.0-fold, respectively (Figure 1B).

**Cathepsin D and estrogen receptor levels in the hamster kidney after treatment with different steroids**

The levels of cathepsin D and estrogen receptor were determined after 5 months of in-vivo treatment with various estrogens, as well as P and DHT (Figure 2). The data show that DES and E2 were the most effective estrogens, inducing 1.8- and 2.0-fold increases in 52 kDa cathepsin D (Figure 2A). In addition, in E2-treated kidneys, both the 27 kDa and, to a lesser extent, the 31 kDa isoforms were present. However, the presence of these latter isoforms were found in lower amounts in DES-treated kidneys; in particular, the 31 kDa-isoform was only faintly detected. A 2.0- and 3.0-fold increase in estrogen receptor levels was also found after DES and E2 treatment, respectively (Figure 2B). The P- and DHT-treated hamsters exhibited no increase in the level of any of the cathepsin D isoforms or in estrogen receptor content when compared to age-matched untreated hamsters.

**Changes in cathepsin D and estrogen receptor content after treatment with Tx alone and in combination with DES**

Cathepsin D and estrogen receptor levels were assessed in hamster kidneys after 5 months of in-vivo treatment with E2, DES, Tx, and Tx + DES, as well as in renal tumors and their metastases from 8 and 9 month DES-treated castrated male hamsters (Figure 3). As shown previously, all cathepsin D isoforms and estrogen receptor levels were elevated in the kidney after 5 months of either DES or E2 treatment alone. However, the rise in 52 kDa cathepsin D and estrogen receptor content seen at 5 months after DES treatment alone, was prevented in hamsters receiving Tx + DES treatment for the same period (Figure 3A and B). Interestingly, treatment with Tx alone, a partial estrogen agonist, did not elicit elevations in either 52 kDa cathepsin D or estrogen receptor content, both of which remained at essentially untreated renal levels. Similarly, when concomitantly administered with DES, DHT
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**Fig. 1.** Representative Western blot analyses of cathepsin D (A) and estrogen receptor (B) during estrogen-induced renal tumorigenesis and in renal tumors. (C) Age-matched untreated controls; (D) DES-treated for various periods of time and (T) renal tumor samples from hamsters receiving DES for 9 and 10 months. The 52 kDa cathepsin D isoform and estrogen receptor levels were markedly elevated at 4 and 5 months of DES treatment when compared to age-matched untreated controls. The increases were more pronounced in primary renal tumors.

completely inhibited the rise in 52 kDa cathepsin D and estrogen receptor content induced by DES treatment alone (data not shown).

Both estrogen-induced primary renal tumors (Figures 1A and 3A) and their metastases (Figure 3A) exhibited appreciable levels of 52 and 27 kDa cathepsin D isoforms and estrogen receptor, when compared to 9 and 10 month age-matched untreated control levels. Although the 52 kDa cathepsin D protein was the major isoform observed in primary hamster renal neoplasms, substantial amounts of the 27 kDa isoform were also present. Moreover, lower levels of the 31 kDa isoform were commonly seen in renal tumors and their metastases (Figures 1A and 3A).

**Localization of cathepsin D during estrogen-induced tumorigenesis**

Cathepsin D was also assessed by immunohistochemical analysis in kidney sections from 4 month DES-treated hamsters, age-matched untreated controls, and 9 month tumor-bearing DES-treated hamsters (Figure 4). Staining was absent in kidney sections from 4 month untreated hamsters (Figure 4A). In contrast, marked cathepsin D activity was consistently found in the cytoplasm of renal proximal tubules after 4 months of estrogen treatment (Figure 4B). Cathepsin D staining in evidently damaged renal tubules of treated animals increased markedly in intensity at 9 months of DES treatment (Figure 4C). The staining in primary renal tumors induced by DES was intense and generally evenly distributed (Figure 4D).

**Discussion**

Estrogen-mediated cytotoxicity found in various tissues following chronic estrogen treatment is well documented, and the subsequent pathologic changes have been described in detail (2,23–25). However, at present there is little understanding of the biochemical and cellular mechanism(s) involved in the cellular damage elicited by chronic exposure to these agents, conditions which frequently lead to a high incidence of tumor formation.

Like the breast and endometrium, the hamster kidney is an estrogen-responsive and -dependent tissue. Therefore, it is not surprising that the estrogen-treated kidney would respond to many of the same genes as the more extensively studied target tissues cited. In this regard, we have shown that c-myc, c-fos, and c-jun, all early estrogen-response genes, are overexpressed in the kidney after only 4 months of estrogen treatment (26). Interestingly, the overexpression of c-myc and c-fos are largely confined to early renal tumorous lesions and tumor foci (27). Cathepsin D is also considered an early estrogen-response gene since its expression appears to operate through an estrogen-responsive element (ERE) in the gene regulator region (28), and is overexpressed in breast cancer. The gene product of cathepsin D is increased in breast cancer (15,17,18) and appears to be mediated by estrogens (15,28). It is noteworthy that the cathepsin D protein has also been found to be elevated in human squamous cervical, colon, and endometrial cancers, the last evidently mediated by progesterone (29). In the hamster
Fig. 2. Representative Western blot analysis of cathepsin D (A) and estrogen receptor (B) after 5 months of treatment with different steroids. (C) Age-matched untreated control. (D) DES. (E2) 17β-estradiol, (E1) estrone. (EE) 17α-ethinylestradiol. (P) Progesterone. (DHT) 5α-dehydrotestosterone. When compared to the levels observed in age-matched controls, only E2 and DES induced appreciable increases (2.0- to 3.0-fold) in the levels of 52 kDa cathepsin D and estrogen receptor. P or DHT treatments for the same length of time had no effect on the levels of either protein.

Fig. 3. Representative Western blot analysis of cathepsin D (A) and estrogen receptor (B) after 5 months of treatment with tamoxifen alone and in combination with DES. (C) Age-matched untreated controls. (D) DES. (E2) 17β-estradiol. (Tx) Tamoxifen. (Tx + D) Tamoxifen plus DES. (Mt) Metastases and (T) renal tumor samples from hamsters receiving DES for 9 and 10 months. The levels of 52 kDa cathepsin D and estrogen receptor were not altered after treatment with Tx alone or in combination with DES. Samples from renal tumor metastasis showed appreciable levels of both proteins.
model, however, cathepsin D protein is absent in the kidneys of untreated hamsters, unlike the c-MYC protein which is found distributed widely in untreated normal renal tubules (27). The 52 kDa-cathepsin D protein is found in appreciable amounts in normal tubules only after estrogen treatment, and along with that of estrogen receptor protein, are both further increased with continued hormone treatment. These findings indicate that cathepsin D gene expression in the hamster kidney is under the control of estrogen and is likely mediated via the induced renal estrogen receptor.

Similar to findings in human breast cancer, benign breast disease, and normal breast tissues (28), the prominent isoform of cathepsin D protein in untreated and estrogen-treated hamster kidneys and primary renal tumors is 52 kDa. There is ample evidence, however, that the major isoform of cathepsin D represents the inactive precursor of the enzyme (32,33). Therefore, it is particularly pertinent that both the 31 kDa and 27 kDa isoforms of cathepsin D, believed to be the processed or active forms of the enzyme (28), are formed only in kidneys of estrogen-treated hamsters, estrogen-induced primary renal tumors, and their metastases.

The data presented herein suggest a novel role for estrogen-induced cathepsin D in the hamster kidney during renal tumorogenesis; that is, mediating renal tubular cell injury as a prelude to reparative cell proliferation, thus initiating a multi-step process of aneuploidy, genomic instability, inappropriate proto-oncogene and suppressor gene expression, gene amplification, and eventual renal tumor development (26,27,34).

Acknowledgements
This paper is dedicated to Dr Gerald Mueller, McArdle Laboratory for Cancer Research, University of Wisconsin, friend, colleague, and muse. We are grateful for the useful editorial comments of Ms Valerie Hahn and for her assistance in the preparation of this manuscript. This investigation was supported by Grants CA 58030 from the National Cancer Institute, NIH, and a grant from the Kansas Masonic Oncology Research Center.

References

Fig. 4. Immunohistochemical staining for cathepsin D with light hematoxylin counterstaining. (A) Untreated hamster kidney from 4 month age-matched controls. Magnification, ×400. (B) Hamster kidney from a 4 month DES-treated hamster kidney. Magnification, ×400. (C) Tumor-bearing kidney from a 9 month DES-treated hamster. Note the granular staining in the cytoplasm of evidently damaged proximal tubules in a nest of renal tumor cells. Magnification, ×800. (D) Large renal tumor from a 9 month DES-treated hamster. Magnification, ×400.

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*Received on February 7, 1997; revised on April 10, 1997; accepted on April 11, 1997*