Estrogen Carcinogenesis in the Hamster Kidney: Role of Cytotoxicity and Cell Proliferation

by Jonathan J. Li,¹ Alfonso Gonzalez,² Snigdha Banerjee,¹ Sushanta K. Banerjee,¹ and Sara Antonia Li¹

Both natural and synthetic estrogens are capable of inducing renal neoplasms in Syrian hamsters with an incidence approaching 100%. Neither the sequence of events nor the mechanisms involved in estrogen carcinogenesis in this model have been established. Results presented here indicate that estrogen induces renal tubular damage in the hamster kidney that is progressive and cumulative. Tubular injury was evident both as abnormal or lost microvilli, accumulation of cytoplasmic lipid droplets, vacuolization, and increases in secondary and tertiary lysosomes after 1.5 months of diethylstilbestrol (DES) treatment. Increasing tubular damage was evidenced by the detachment of tubular cells, cell debris, and occluded renal tubular lumens. In an effort to repair proximal tubular damage in the hamster kidney elicited by estrogens, a 4.0-fold increase in proximal tubule BrdU labeling was evident at 4 months of DES or 17β-estradiol (E2) treatment and in earlier estrogen treatment periods (1-3 months). During this period, there was a significant increase in aneuploid cells in the hamster kidney, the near diploid frequency increased more than 6.0-fold, and the near tetraploid frequency increased at least 3.0-fold between 1.5 and 3.5 months of estrogen treatment. Based on these data, the early sequence of events leading to estrogen-induced renal neoplastic transformation in the hamster is presented.

Introduction

Estrogens have been associated as causative agents in tumor development for over half a century, and despite intensive investigation, whether hormonal agents behave as genotoxic or epigenetic carcinogens continues to be debated. The estrogen-induced kidney tumor in the hamster is one of the most investigated experimental models in hormonal carcinogenesis. Despite this, little is known either about the sequence of events or mechanisms involved in the estrogen carcinogenicity of this and other hormone-induced animal cancers. Based on our studies, we have proposed that estrogens are nongenotoxic (epigenetic) carcinogens. We define nongenotoxic carcinogens as agents that do not act directly or indirectly with genetic material but nevertheless are capable of leading to heritable changes in the structure or sequence of the genetic material at the level of the nucleic acid, the gene, or the chromosome by alternative mechanisms.

Multiple, bilateral renal tumors can be induced by either natural and synthetic steroidal or stilbene estrogens in intact or castrated male Syrian hamsters, with an incidence near 100% (1). It is relevant that spontaneous renal tumors in the hamster are essentially nonexistent (2-4). Evidence is beginning to emerge from our laboratory on the general nongenotoxic sequence of events leading to estrogen-induced neoplastic transformation in the hamster kidney (5). This paper describes the early events in estrogen carcinogenesis of the hamster kidney based on data from our laboratory.

¹Hormonal Carcinogenesis Laboratory and Department of Pharmaceutical Sciences, College of Pharmacy, Washington State University, Pullman, WA 99164-6510.
²Department of Pathology and Laboratory Service, University of Utah and Veterans Administration Medical Center, Salt Lake City, UT 84132.

Address reprint requests to J. J. Li, Hormonal Carcinogenesis Laboratory and Department of Pharmaceutical Sciences, College of Pharmacy, Washington State University, Pullman, WA 99164-6510.

This paper was presented at the Symposium on Cell Proliferation and Chemical Carcinogenesis that was held January 14-16, 1992, in Research Triangle Park, NC.
Methods

Animals and Treatment

Castrated, young adult male Syrian golden hamsters, noninbred, weighing 85-90 g, were purchased from Harlan Sprague-Dawley (Indianapolis, IN). Hamsters were exposed to 12-hr light: dark cycles, fed rodent certified chow (5002 Purina diet), and given tap water ad libitum. Hormone pellets containing either diethylstilbestrol (DES) or 17β-estradiol (E2) were implanted sc in the treated group as described previously (6). To maintain constant levels of hormone, new pellets were implanted every 2.5 months. Age-matched control animals received no hormone implants. Groups of hamsters from the treated and age-matched controls were killed at monthly intervals after the initial hormone pellet implant. The mean daily absorption of estrogen in the treated groups did not differ appreciably from values previously reported (7).

Light and Electron Microscopy

All kidneys were prepared by a whole-body fixation perfusion technique (6) using 2% glutaraldehyde prewarmed to 37°C. The kidneys were separated and serially cut into 1-mm slices. Whole slices were processed for light and electron microscopy. Light microscopic examination was performed on paraffin-embedded sections and stained with hematoxylin and eosin. Kidney tissue prepared for electron microscopy was fixed in 2% glutaraldehyde in Sorensen’s phosphate buffer, pH 7.4, and postfixed in Caulfield’s 2% osmium tetroxide. After dehydration in a graded series of ethanol with 100% propylene oxide as a transitional solvent, the tissues were embedded in Epon 812. Thin sections were cut with an LKB ultramicrotome, stained with lead citrate and uranyl acetate, and examined using a Hitachi H-300 electron microscope. Details of these procedures have been described elsewhere (9).

Labeling Index

Hamsters were implanted with mini-osmotic pumps (Alzet Model 2001), for 1 week at a concentration of 20 mg/mL of bromodeoxyuridine (BrdU; 0.1 mg/g body weight; Alzet Corporation, Palo Alto, CA). Detection of BrdU-labeled cells was based on the procedure by Vector Laboratories ABC Kit with slight modification. Briefly, kidneys were excised, the capsule removed, and placed in Tissue-TEK (OCT compound, Miles, Inc., Elkhart, IN), and frozen at −20°C. Tissues were sectioned at 5 μm and mounted on microscopics slides. Before staining, sections were air dried, fixed in 4% formalin for 10 min, hydrated, and incubated in 1 N HCl for 60 min at 40°C. After incubation, the sections were washed in phosphate-buffered saline (PBS), pH 7.6, and treated with 1% hydrogen peroxide in methanol for 20 min. The slides were then incubated sequentially in blocking solution (normal horse serum) for 20 min, primary monoclonal antibody anti-BrdU (Becton-Dickinson, San Jose, CA) at a concentration of 0.12 μg/mL for 3 hr, biotinylated secondary antibody diluted in PBS for 30 min, and ABC complex for 45 min. After each incubation, the slides were washed with PBS. Finally, the sections were stained with diaminobenzidine (DAB) and counter-stained with hematoxylin, mounted, and examined under the microscope. Control slides were incubated with normal mouse IgG or without primary antibody. Bromodeoxyuridine (BrdU) incorporation was assessed in each kidney using at least 30 serial transverse sections from each tissue block. The labeling index (LI) represents the percentage of labeled cells divided by the total number of cells × 100. For the present studies, at least 5000 cells were counted in each section.

Chromosomal Analysis

In the preparation of metaphase chromosomes, cultured proximal tubules or cortical kidney fragments were treated with colchicine (0.4 μg/mL) for 3–4 hr. Kidney cells were trypsinized and then treated with hypotonic solution (0.075 M KCl) for 30 min at 37°C. The suspended cells were centrifuged, and the pellets were fixed in cold acetic acid: methanol (3:1) solution. Slides containing chromosomes were air dried and stained with 2% Giemsa solution. One hundred metaphases from each group were analyzed for aneuploidy.

Results

Cytotoxicity

Renal tubular damage was observed in hamsters after chronic estrogen (DES or E2) exposure. The severity of the tissue damage increased progressively with prolonged hormone exposure. The proximal convoluted tubules were the most affected, followed by the distal convoluted tubules and collecting ducts. Tubular injury was seen both in abnormal microvilli and losses in surface microvilli, accumulation of lipid droplets in the cytoplasm, and marked increases in numerous secondary and tertiary lysosomes (Fig. 1B, C), none of which was seen in normal, untreated tubules (Fig. 1A). In addition, numerous clear or empty vacuoles were evident in the renal tubular cells after chronic estrogen exposure. This may result from distended endoplasmic reticulum. Binucleated cells were also commonly observed in treated cells but were absent in untreated renal tubular cells. (Fig. 1B). Detached tubular cells, cell debris, and partially or totally occluded renal tubular lumens were also found after prolonged estrogen treatment. In severely damaged tubular cells, the cytoplasm was swollen and projected into the tubular lumen as lighter, relatively empty cytoplasmic globules, which often resulted in complete obliteration of the lumen (Fig. 1C). Glomerular
**Figure 1.** (A) Electron micrograph of untreated hamster kidney from castrated, adult male. Two adjacent renal tubules, a proximal convoluted tubule (PCT) with typical long microvilli and a distal convoluted tubule (DCT) with shorter, rounded microvilli are shown. Both PCT and DCT exhibit very few lysosomes (arrows). Cytoplasmic elements appear normal, 36,000x. (B) Electron micrograph of hamster kidney treated with diethylstilbestrol (DES) for 1 month. Two PCT are seen separated by a capillary. Numerous dark lysosomes and clear vacuoles are evident in every cell. The cell in the lower left corner is binucleated, 2000x. (C) Electron micrograph of hamster kidney treated with DES for 5 months. Increased PCT damage is reflected by numerous lysosomes, vacuoles, and binucleation. The lower left PCT exhibits abnormal microvilli, and other PCT have either fragmented or lost their microvilli. Swollen cytoplasm frequently projects into the tubular lumen. 1000x.
damage was minimal after relatively brief periods of estrogen exposure. After 7–8 months of treatment, however, glomerular damage was evident by the effacement of epithelial cell foot processes, by an increase in mesangial matrix, and in some cases progression to total glomerular sclerosis.

**Cell Proliferation**

**BrdU Labeling.** BrdU-labeled kidney cells were relatively infrequent (1.7–2.6%) in untreated hamsters in the different age-matched groups. After 4 months of DES treatment, BrdU-labeled renal cells increased 4.0-fold in each kidney. The labeled renal cells were almost exclusively found in mature proximal renal tubules (Fig. 2). The increased number of BrdU-labeled renal cells in each kidney was essentially the same. However, the left kidney displayed slightly higher labeling indexes compared to the right kidney and compared to corresponding unexposed hamster kidneys. With continued estrogen exposure for 7 months, the percentage of BrdU-labeled cells significantly ($p < 0.05$) increased about 7.0-fold in each kidney compared to kidneys of untreated, age-matched animals.

**Aneuploidy.** In untreated hamsters, the majority of kidney metaphase chromosomes exhibited 2n=44 chromosomes (>90%). The frequency of near diploid (between 40 and 43 and 45 and 48) was 6%, and the near tetraploid frequency (>51) was less than 2% in normal control kidney (Fig. 3) Early stages of either E$_2$-or DES-treated hamsters indicated a similar rise in near diploid frequency to 38–39% and a lesser increase in the near tetraploid frequency (6–7%) after 1.5 and 3.5 months of estrogen treatment (Fig. 3). The percentage of aneuploid proximal tubular cells in DES-treated hamsters remained elevated at the same level after 9 months of hormone treatment (Fig. 4).

**Discussion**

Regenerative or reparative hyperplasia is a characteristic response of many nongenotoxic cytotoxic agents in the kidney (10–12). Interestingly, these xeno-biotic nephrotoxicants are also kidney carcinogens in male but not female rats. It has been suggested that this sequence of events, in which cell proliferation has a conspicuous role, is the driving force in the induction of these tumors (13). It therefore may be surprising to some that excessive hormone exposure (i.e., estrogens) may have a similar sequence of initial events that then set the stage for the occurrence of aneuploidy and other chromosomal abnormalities in the hamster kidney.

A revised scheme (15) for the sequence of events leading to estrogen-induced renal tumorigenesis in the hamster kidney is presented in Figure 5. There are two events occurring at approximately the same time. First, there is alteration in renal proximal tubule (RPT) cells, which is manifested by an elevation in both estrogen and progesterone receptors (14). This clearly suggests an increased responsiveness of the
kidney tubule to estrogen. Second, there is progressive RPT cytotoxicity and cell damage with continued estrogen exposure, which is detectable as early as 1.5 months. This cell damage, which may arise from hormonal and perhaps nonhormonal mechanisms, increases in severity with continued hormone treatment. Free radicals may be generated as a result of this accumulated cell damage, thus facilitating the cytotoxic process. It is conceivable that ascorbic acid (vitamin C), shown to partially reduce renal tumor incidence (15), may act at this point by inhibiting the renal tubular damage induced by estrogen. Initially, when the tubular damage is not severe, a reparative hyperplasia occurs, and this is seen in the BrdU labeling of mature proximal tubule cells. With increased severity in tubular cell damage, multipotential primitive interstitial stem cell populations, shown to be the origin of this tumor (9,20), begin to proliferate in an effort to repair the accumulating cell damage induced by chronic estrogen exposure (data not shown). This specific estrogen-induced cell proliferation has been observed in normal hamster proximal tubules in vitro grown on HR-9 or MDCK matrices under serum-free, chemically defined conditions (17). As a consequence of this regenerative cell proliferation, both in the mature proximal tubule (limited) and primitive interstitial stem cells, aneuploid cells are greatly increased. These data strongly suggest the possibility for the existence of nonrandom chromosomal gains (trisomies, tetrasomies) and losses (monosomies) as early critical events in renal tumorigenesis. Consequently, there may be specific regions of low-level gene amplification and enhanced expression of cellular oncogenes (18) as well as sites of suppressor gene function that may ultimately effect neoplastic transformation in the hamster kidney in a discrete sequence of molecular events.

![Figure 4](image-url)  
**Figure 4.** Comparison of the percentage of aneuploidy in hamster kidney cells after 1.5, 3.5 and 9.0 months diethylstilbestrol (DES) treatment. It should be noted that the latter treatment period represents a mixture of tumor and normal cell populations. The early two DES treatment periods represent nontumorous, chronically estrogenized kidneys. Aneuploid frequency attained a maximal elevated level between 3.0 and 9.0 months of continuous DES treatment. Values represent the means ± SE (bars) of at least five to seven individual determinations.

![Figure 5](image-url)  
**Figure 5.** Scheme for sequence of events leading to estrogen-induced renal tumorigenesis in hamsters.
This study was supported by grants CA 22009 and CA 41267 (Shannon Award) from the National Cancer Institute and an American Cancer Society Institutional Research Grant, Washington State University.

REFERENCES

1. Kirkman, H., and Bacon, R. L. Malignant renal tumors in male hamster (Cricetus auratus) treated with estrogens. Cancer Res. 10: 122–124 (1950).

2. Kirkman, H. Estrogen-induced tumors of the kidney. Natl. Cancer Inst. Monogr. 1: 1–5 (1959).

3. Pour, P., Mohr, U., Althoff, J., Cardesa, A. and Kmoch, M. Spontaneous tumors and common diseases in two colonies of Syrian hamsters. III. Urogenital system and endocrine glands. J. Natl. Cancer Inst. 56: 949–961 (1976).

4. Pour, P., Althoff, J., Salmasi, S. Z., and Stephen, K. Spontaneous tumors and common diseases in three types of hamsters. J. Natl. Cancer Inst. 63: 797–811 (1979).

5. Li, J. J., and Li, S. A. Estrogen carcinogenesis in hamster tissues: A critical review. Endocr. Rev. 11: 524–531 (1990).

6. Li, J. J., Kirkman, H., and Hunter, R. L. Sex difference and gonadal hormone influence on Syrian hamster kidney esterase isozymes. J. Histochem. Cytochem. 17: 386–395 (1969).

7. Li, J. J., Li, S. A., Klicka, J. K., Parsons, J. A., and Lam, L. K. T. Relative carcinogenic activity of various synthetic and natural estrogens in the Syrian hamster kidney. Cancer Res. 43: 5200–5204 (1983).

8. Sjostrand, F. S. The preparation of specimens by chemical fixation. Fixation by perfusion. In: Electron Microscopy of Cells and Tissues, Vol. 1. Instrumentation and Techniques F. S. Sjostrand, Ed.) Academic Press, New York, 1967, pp. 155–176.

9. Gonzalez, A., Oberley, T. D., and Li, J. J. Morphological and immunohistochemical studies of the estrogen-induced Syrian hamster renal tumor. Probable cell of origin. Cancer Res. 49: 1029–1028 (1989).

10. Short, B. G., Burnett, V. L., and Swenber, J. A. Histopathology and cell proliferation induced by 2,2,4-trimethylpentane in the male rat kidney. Toxicol. Pathol. 14: 194–203 (1986).

11. Loury, D. J., Smith-Oliver, T., and Butterworth, B. E. Assessment of unscheduled and replicative DNA synthesis in rat kidney cells exposed in-vitro or in-vivo to unleaded gasoline. Toxicol. Appl. Pharmacol. 87: 127–140 (1987).

12. Goldsworthy, T. L., Lyght, O., Burnett, U. L., and Popp, J. A. Potential role of α-2u-globulin, protein droplet accumulation and cell replication in the renal carcinogenicity of rats exposed to trichloroethylene, perchloroethylene, and pentachloroethane, Toxicol. Appl. Pharmacol. 96: 367–379 (1988).

13. Butterworth, B. E. Nongenotoxic carcinogens in the regulatory environment. Regul. Toxicol. Pharmacol. 9: 244–256 (1989).

14. Li, J. J., and Li, S. A. Estrogen-induced tumorigenesis in hamsters: Role for hormonal and carcinogenic activities. Arch. Toxicol. 55: 110–118 (1984).

15. Liehr, J. G., and Wheeler, W. J. Inhibition of estrogen-induced renal carcinomas in Syrian hamsters by vitamin C. Cancer Res. 43: 4638–4642 (1983).

16. Oberley, T. D., Gonzalez, A., Lauchner, L. J., Oberley, L. W., and Li, J. J. Characterization of early kidney lesions in estrogen-induced tumors in the Syrian hamster. Cancer Res. 51: 1922–1924 (1991).

17. Oberley, T. D., Lauchner, L. J., Pugh, T. D., Gonzalez, A., Goldfarb, S., Li, S. A. and Li, J. J. Specific estrogen-induced cell proliferation of cultured Syrian hamster renal proximal tubular cells in serum-free chemically defined media. Proc. Natl. Acad. Sci., U. S. A. 86: 2107–2111 (1989).

18. Solomon, E., Borrow, J., and Goddard, A. D. Chromosome aberrations and cancer. Science 254: 1153–1160 (1991).