Combined Histochemical and Biochemical Analysis of Sex Hormone Dependence of Ferric Nitrilotriacetate-induced Renal Lipid Peroxidation in ddY Mice

Shinya Toyokuni, Shigeru Okada, Shuji Hamazaki, Yukiko Minamiyama, Yoshihiro Yamada, Ping Liang, Yasutomo Fukunaga, and Osamu Midorikawa

Department of Pathology, Faculty of Medicine, Kyoto University, Sakyo-ku, Kyoto 606, Japan

ABSTRACT

Ferric nitrilotriacetate (Fe-NTA) induces renal proximal tubular necrosis, a consequence of lipid peroxidation, that finally leads to a high incidence of renal adenocarcinoma in rats and mice. Male animals are much more susceptible than female animals to both effects. Moreover, the distribution of the susceptible proximal tubules is different between male and female animals. The present study investigated the effects of castration and sex hormones on Fe-NTA-induced renal lipid peroxidation. Male and female ddY mice were either left untreated, castrated, and/or treated with testosterone or estradiol. Histochemical (reactivity to cold Schiff's reagent) and biochemical (thiobarbituric acid-reactive substance) evaluations were performed 1 h after the i.p. injection of Fe-NTA (5 mg iron/kg).

Testosterone treatment and/or oophorectomy increased the Schiff positivity of the renal cortical proximal tubules and the amount of thiobarbituric acid-reactive substance (testosterone-treated female > intact female, \( P < 0.005 \); castrated female > intact female, \( P < 0.1 \); castrated and testosterone-treated female > intact female, \( P < 0.005 \)). In contrast, estradiol treatment and/or oophorectomy decreased the Schiff positivity of the renal cortical proximal tubules and the amount of thiobarbituric acid-reactive substance (estradiol-treated male < intact male, \( P < 0.01 \); castrated male < intact male, \( P < 0.01 \); castrated and estradiol-treated male < intact male, \( P < 0.005 \)). Estradiol treatment produced similar results to estradiol treatment (estradiol-treated male < intact male, \( P < 0.005 \)). Castration and/or administration of the opposite sex hormone reversed the sex difference in the distribution of proximal tubules susceptible to lipid peroxidation. However, the i.v. injection to male mice, 5 min prior to the Fe-NTA treatment, of conjugated estrogen that is promptly excreted via the urine produced no significant effect. Thus, altered metabolic pathways rather than the direct scavenging activity of estrogens seem to be involved in the sex hormone-dependent difference of lipid peroxidation. Genetically determined sex hormone status appears to have influenced the incidence of Fe-NTA-induced renal adenocarcinoma in intact animals.

INTRODUCTION

NTA\(^1\) is a synthetic aminopolyacetic acid that efficiently forms water-soluble chelate complexes with several metal cations at a neutral pH and has been used as a substitute for polyphosphates in detergents for household and hospital use in the United States and Canada (1, 2). Experimental models of iron overload (3) and copper toxicity (4) have been developed by using Fe-NTA and cupric NTA, respectively. We have hitherto reported the occurrence of acute and subacute renal proximal tubular necrosis and a subsequent high incidence of renal adenocarcinoma in rats and mice given repeated i.p. injections of Fe-NTA (5–10). In this model male mice are much more susceptible than female mice to both acute and subacute nephrotoxicity and the carcinogenic effect of Fe-NTA (10, 11), which causes lipid peroxidation in the renal proximal tubules (11–14). Lipid peroxidation appears to play an essential role in carcinogenesis, because i.p. aluminum-NTA administration, which induces acute and subacute renal proximal tubular necrosis without lipid peroxidation, leads to no renal neoplasms (9, 12). We have recently found differences between male and female mice in the vulnerable segment and the severity of Fe-NTA-induced renal lipid peroxidation.\(^4\) In the present study, we investigated the effects of castration and sex hormone treatment in a simplified model with single i.p. injections of Fe-NTA.

MATERIALS AND METHODS

Animals, Castration, and Hormone Treatment. Male and female ddY mice (8–9 weeks of age; males, 32–34 g; females, 27–29 g; Shizuoka Laboratory Animal Center, Shizuoka, Japan) were housed in clear plastic cages and fed a basal diet (F-1; Funabashi, Japan) and soft water ad libitum. A total of 71 male and 51 female mice were used. Ninety of them were divided into 10 groups of 10 or 5 groups (1 and 6) animals (Table 1). The animals of groups 4, 5, 9, and 10 were castrated under pentobarbital anesthesia within 1 week after they were purchased and were kept untreated for at least 4 weeks before the next procedure. The animals of groups 3, 5, 8, and 10 were given a s.c. injection of either 10 mg/kg testosterone propionate (Testinon; Mochida, Tokyo, Japan) or 1 mg/kg estradiol suspension (Estril; Mochida) twice a week for 2 weeks before the lipid peroxidation experiment. Five animals of each group (except groups 1 and 6) were used solely for testosterone and estradiol assays. Six male and 6 female mice were used for histological evaluation after the injection of Fe-NTA. The other 20 male animals were used for the evaluation of estradiol and i.v. water-soluble conjugated estrogen treatment. Five mg/kg estradiol valerate (Pelanin Depot; Mochida) was injected i.m. once a week for 2 weeks. Four mg/kg conjugated estrogen (principally estrone sodium sulfate and equilin sodium sulfate: Romeda; Mochida), which is immediately excreted in the urine as estrone, estradiol, or estril, was injected i.v. in the tail vein.

Preparation and Injection of Fe-NTA Solution and Histological Examination. The Fe-NTA solution was prepared immediately before use by the method of Awai et al. (3), which was modified. Ferric nitrate enneahydrate (Ishizu, Osaka, Japan) and nitrilotriacetic acid disodium salt (Nakarai, Kyoto, Japan) were each dissolved in deionized water by the method of Awai et al. (3), which was modified. Ferric nitrate was prepared immediately before use by the method of Awai et al. (3), which was modified. Ferric nitrate enneahydrate (Ishizu, Osaka, Japan) and nitrilotriacetic acid disodium salt (Nakarai, Kyoto, Japan) were each dissolved in deionized water by the method of Awai et al. (3), which was modified.

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1 This work was supported in part by a Grant-in-Aid from the Ministry of Health and Welfare, Japan.

2 To whom requests for reprints should be addressed.

3 The abbreviations used are: NTA, nitrilotriacetic acid; Fe-NTA, ferric nitrilotriacetate; TBARS, thiobarbituric acid-reactive substance; H&E, hematoxylin and eosin.

SEX HORMONES AND RENAL LIPID PEROXIDATION

Table 1 Experimental groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Castration</th>
<th>Estriol</th>
<th>Fe-NTA</th>
<th>Group</th>
<th>Castration</th>
<th>Estriol</th>
<th>Fe-NTA</th>
</tr>
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<tbody>
<tr>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>6</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>7</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>8</td>
<td>-</td>
<td>+</td>
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<td>9</td>
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</tr>
<tr>
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<td>+</td>
<td>+</td>
<td>10</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

*-, not treated; +, treated.

Table 2 Histochemical detection of lipid peroxidation in the renal proximal tubules

<table>
<thead>
<tr>
<th>Group</th>
<th>Cortex</th>
<th>Outer stripe</th>
<th>Group</th>
<th>Cortex</th>
<th>Outer stripe</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control†</td>
<td>-</td>
<td>-</td>
<td>Control</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Fe-NTA</td>
<td>++</td>
<td>-</td>
<td>T Fe-NTA</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>E Fe-NTA</td>
<td>+</td>
<td>+</td>
<td>Cast Fe-NTA</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Cast E Fe-NTA</td>
<td>-</td>
<td>+</td>
<td>Cast Fe-NTA</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

† Control, untreated control; E, estriol-treated; T, testosterone-treated; Cast, castrated; -, negative staining; +, positive staining; ++, strongly positive staining; ++++, intensely positive staining.

at −40°C for the histochemical detection of lipid peroxidation. The other was chilled in ice-cold physiological saline solution for the TBARS determination. Six male and 6 female mice were killed at either 1 or 3 h, and H&E staining after 10% neutral formalin fixation was used for the light microscopic observations.

Histochemical Detection of Lipid Peroxidation. Histochemical detection of lipid peroxidation was performed within 3 days after sacrifice, using cold Schiff's reagent, by the method of Pompella et al. (15).

Measurement of TBARS. The tissue TBARS content was determined immediately after sacrifice, using the method of Okawa et al. (16) after modification. The pelvis of each kidney was excised before homogenization. We added 0.1% 2,6-di-tert-butyl-p-cresol (Nacalai Tesque Inc., Kyoto, Japan) to the reaction mixture to prevent additional chromophore formation during the assay procedure (17). Protein determination was performed by the method of Lowry et al. (18), using bovine serum albumin (Sigma, St. Louis, MO) as a standard.

Testosterone and Estriol Assays. Blood was obtained under pentobarbital anesthesia by aortic puncture. Serum was separated immediately and stored frozen at −40°C until determination. Serum testosterone and estriol levels were, respectively, assayed by using the 125I-testosterone radioimmunoassay kit EIKEN (EIken Kagaku, Tokyo, Japan) and the radioimmunoassay kit Total E, RIA Pack II (Amersham Japan, Tokyo, Japan), with an ARC-950 γ-counter (Aloka Co. Ltd., Tokyo, Japan).

Statistical Analysis. Statistical analyses were performed by the unpaired t test, which was modified for unequal variances when necessary.

RESULTS

Histochemical Detection of Lipid Peroxidation and Histological Findings. The histochemical findings are summarized in Table 2. Kidneys from untreated male and female mice (groups 1 and 6) were unstained after exposure to cold Schiff's reagent, except for the elastic fibers of arterial walls. One h after the Fe-NTA injection, male mice (group 2) showed patchy Schiff positivity in the renal proximal tubules (Fig. 1), at which time routine histological examination with H&E staining showed no remarkable changes. At 3 h, however, patchy degeneration of the cortical proximal tubular epithelium could be observed, with pyknotic nuclei and necrotic debris in the tubules (Fig. 2).

In contrast, female mice 1 h after the Fe-NTA injection (group 7) showed weaker and limited Schiff positivity of the outer stripe of the outer medulla, which also consisted of proximal tubules (19), and no Schiff positivity in the cortical proximal tubules (Fig. 3). H&E-stained sections showed no significant changes either at 1 or 3 h (Fig. 4).

Estriol treatment and/or orchiectomy decreased the Schiff
Fig. 3. Female mouse 1 h after Fe-NTA injection. Diffuse patchy Schiff-positive areas in the outer stripe of the outer medulla (asterisk). × 40.

Fig. 4. Female mouse 3 h after Fe-NTA injection. No significant changes in the cortex. H&E. × 200.

Fig. 5. Estriol-treated male mouse 1 h after Fe-NTA injection. Weak patchy Schiff positivity in the cortical proximal tubules and faint Schiff positivity of the outer stripe of the outer medulla (asterisk). × 40.

Fig. 6. Castrated male mouse 1 h after Fe-NTA injection. Dispersely distributed Schiff positivity in the cortical proximal tubules and faint Schiff positivity of the outer stripe of the outer medulla (asterisk). × 40.

Sex hormones and renal lipid peroxidation

positivity of the renal cortical proximal tubules but increased the Schiff positivity of the outer stripe of the outer medulla (groups 3–5) (Figs. 5–7). Estradiol treatment revealed the same effect as estriol treatment (data not shown). In contrast, testosterone treatment and/or oophorectomy increased the Schiff positivity of the renal cortical proximal tubules but decreased the Schiff positivity of the outer stripe of the outer medulla (groups 8–10) (Figs. 8–11). Schiff-positive material appeared in the proximal tubules just after the glomerular filtration, and glomeruli and some of the cortical proximal tubules were com-
SEX HORMONES AND RENAL LIPID PEROXIDATION

Fig. 7. Castrated and estriol-treated male mouse 1 h after Fe-NTA injection. No Schiff positivity in the cortical proximal tubules and faint Schiff positivity of the outer stripe of the outer medulla (asterisk). × 40.

Fig. 9. Castrated female mouse 1 h after Fe-NTA injection. Dispersely distributed Schiff-positive areas in the cortical proximal tubules and faint Schiff positivity of the outer stripe of the outer medulla (asterisk). × 40.

Fig. 8. Testosterone-treated female mouse 1 h after Fe-NTA injection. Strong patchy Schiff positivity of the cortical proximal tubules. × 40.

Fig. 10. Castrated and testosterone-treated female mouse 1 h after Fe-NTA injection. Very strong patchy Schiff positivity in the cortical proximal tubules. × 40.

pletely spared in either group (Fig. 11).

Measurement of TBA-reactive Substance. Testosterone treatment and/or oophorectomy increased the TBARS content (testosterone-treated female > intact female, \( P < 0.005 \); castrated female > intact female, \( P < 0.1 \); castrated and testosterone-
treated female > intact female, \( P < 0.005 \)). In contrast, estriol treatment and/or orchietomy decreased the TBARS content (estriol-treated male < intact male, \( P < 0.01 \); castrated male < intact male, \( P < 0.01 \); castrated and estriol-treated male <
intact male, \( P < 0.005 \) (Table 3, Fig. 12). Male mice treated with estradiol (58.5 ± 9.5 nmol malondialdehyde/100 mg protein; mean ± SE; \( P < 0.005 \) versus intact male; \( n = 5 \)) showed similar results as those treated with estriol. Intravenous pre-treatment with water-soluble conjugated estrogen (134.9 ± 22.6, \( n = 5 \)) produced no significant change in the amount of TBARS in comparison with animals without pre-treatment (126.4 ± 23.0 nmol malondialdehyde/100 mg protein; mean ± SE; \( n = 5 \)).

Assay of Testosterone and Estriol. Serum levels of each sex hormone in animals of groups 2–5 and 7–10 at sacrifice are shown in Table 4.

**DISCUSSION**

Castration and/or treatment with the opposite sex hormone reversed the sex differences in the distribution and severity of lipid peroxidation in the renal proximal tubules in this murine model.

It is known that androgen and estrogen receptors exist in the renal proximal tubules (20–22). It has been reported that the expression of several genes (23, 24) and the incorporation of uridine into RNA (25) are regulated by androgens in the murine kidney. The differences between male and female mice in the distribution and activity of several enzymes (26, 27) as well as microscopical morphological differences (26–30) have also been reported in relation to sex hormones.

It was of note that not only estradiol but also estriol had a dramatic inhibitory effect on lipid peroxidation in this model. This is consistent with the recent finding that pregnancy, during which estriol shows the most prominent increase among estrogens (31), lowers renal and hepatic lipid peroxidation (32, 33). It has also been reported that estrogens itself (estrone, estradiol, and estriol), but not testosterone, is an antioxidant in vitro (34–36). We believe, however, that a role for estrogen itself as an antioxidant is unlikely in this model for the following reasons. Firstly, there was a change in the distribution of Fe-NTA-induced lipid peroxidation of the renal proximal tubules between male and female mice (Figs. 1 and 3). Secondly, testosterone markedly promotes lipid peroxidation, as shown in Figs. 8, 10, and 12. Thirdly, an increase in the estrogen content of the renal tubular lumens by i.v. estrogen administration had no significant effect on the TBARS content. Fourthly, the estrogen-rich environment attained in vitro (34–36) seems unlikely to be obtained in the luminal membrane of the renal proximal tubules in vivo when the total amount of estrogen administered is considered. Finally, in another model of kidney damage associated with lipid peroxidation produced by phenylbutazone, female rats are more susceptible than male animals (37).

We have recently reported, in in vitro experiments, that the reduction from ferric to ferrous state and the immediate further autoxidation was essential for the induction of oxygen radicals by Fe-NTA, and we suggested that lipid peroxidation was initiated at the luminal membrane of the proximal tubules in this model (8, 38, 39). This was confirmed by our present study in which Schiff-positive material appeared in the proximal tubules just after the glomerular filtration (Fig. 11). We thus speculate that either functional heterogeneity in the ability to reduce iron in the proximal tubular lumen, the distribution of antioxidants such as vitamin E (40, 41), superoxide dismutase, glutathione (42, 43), and metallothionein (44), or the renal tubular localization of unsaturated lipids that provide the major substrates for lipid peroxidation is closely associated with the sex hormones.

We demonstrated by modifications of sex hormones that the susceptibility of renal proximal tubules to lipid peroxidation by this iron chelate was genetically determined. The data from both biochemical quantitative detection and histochemical localization supported this conclusion. These findings seem strongly connected to the difference in carcinogenesis between male and female animals (10). An experiment assessing the effects of castration on Fe-NTA-induced renal carcinogenesis is now in progress.

In this model TBARS reaches the maximum value from 30 min to 2 h after the administration, followed by cortical proximal tubular necrosis at no later than 3 h and regeneration thereafter. In the case of repeated administration, morphologically atypical regenerative cells with positive iron staining appear as early as at 10 days, and atypical cells and atypia itself increase according to the experimental days (5–7, 9, 10). We also observed the process from dysplastic cells to carcinoma, as is seen in estrogen-induced renal adenocarcinoma of hamsters (45). The exact significance of lipid peroxidation, whether it is solely a concomitant of oxygen radical-induced DNA damage that may lead to carcinogenesis (46) or whether its products increase according to the experimental days (5–7, 9, 10). We also observed the process from dysplastic cells to carcinoma, as is seen in estrogen-induced renal adenocarcinoma of hamsters (45). The exact significance of lipid peroxidation, whether it is solely a concomitant of oxygen radical-induced DNA damage that may lead to carcinogenesis (46) or whether its products increase according to the experimental days (5–7, 9, 10). We also observed the process from dysplastic cells to carcinoma, as is seen in estrogen-induced renal adenocarcinoma of hamsters (45). The exact significance of lipid peroxidation, whether it is solely a concomitant of oxygen radical-induced DNA damage that may lead to carcinogenesis (46).
role in mutagenicity (47) or initiation (49), requires further study to be elucidated.

The histochemical detection of lipid peroxidation is helpful in interpreting the significance of borderline data obtained by biochemical studies, like that for the castrated female mice (Figs. 3, 9, and 12). The precise mechanism of sex hormone function in carcinogenesis in this model should be further investigated, since the carcinogenic process is completed in a relatively short period of time (10) with male preponderance.

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The authors thank Dr. Yoshiro Niitsu (Department of Internal Medicine, Sapporo Medical College, Sapporo, Japan) for advice and encouragement and Yoshinobu Toda, Hitoshi Abiru, Makio Fujioka, Shinji Toh, and Hisae Yorisawa for the technical assistance.

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ANNOUNCEMENTS

GENOMIC INSTABILITY AND CANCER
February 21–26, 1994
Banff Springs Hotel,
Banff, Alberta, Canada

Chairpersons
Thea D. Tlsty, Chapel Hill, NC
Lawrence A. Loeb, Seattle, WA

GROWTH FACTORS, DEVELOPMENT,
AND CANCER
(Joint Meeting with the Friedrich Miescher-Institut)
March 5–11, 1994
Congress Center, Interlaken, Switzerland

Chairpersons
Harold L. Moses, Nashville, TN
Bernd Groner, Basel, Switzerland

RECENT DEATHS
We regret to report the deaths of Dr. Zalmen A. Arlin of the New York Medical College, Valhalla, NY; Dr. M. Adrian Gross of Silver Spring, MD; Dr. Arthur Worth Ham of Markham, Ontario, Canada; Dr. S. Charles Kasdon of Boston, MA; and Dr. Einar Pedersen of The Norwegian Radium Hospital, Oslo, Norway. Drs. Arlin and Gross were Active Members, Dr. Pedersen was a Corresponding Member, and Drs. Ham and Kasdon were Emeritus Members of the American Association for Cancer Research.

CALENDAR OF EVENTS


Arizona Cancer Center Fifth International Workshop on Chromosomes in Solid Tumors, January 10–12, 1993, Doubletree Inn, Tucson, AZ. Contact: Nancy Rzewuski, Arizona Cancer Center, 1515 North Campbell Avenue, Tucson, AZ 85724. Telephone: (602) 626-6044; FAX: (602) 626-2284.

Basal and Squamous Cell Head and Neck Skin Cancer—Partners in Excellence Series, February 6, 1993, M. D. Anderson Cancer Center, Houston, TX. Contact: Carol Harreld, Conference Services, Box 131, 1515 Holcombe Boulevard, Houston, TX 77030-4095. Telephone: (713) 792-2222; FAX: (713) 794-1724.

Seventh International Conference on the Adjuvant Therapy of Cancer, March 10–13, 1993, Tucson Convention Center, Tucson, AZ. Abstract deadline: December 1, 1992. Contact: Nancy Rzewuski, Arizona Cancer Center, University of Arizona College of Medicine, 1515 N. Campbell Avenue, Room 2933, Tucson, AZ 85724. Telephone: (602) 626-2276; FAX: (602) 626-2284.

Women's Health and Breast Cancer—A Continuing Education Course, March 13, 1993, University of California, Davis, Medical Center, Sacramento, CA. Credits: 6 hours Category 1 AMA/CMA. Contact: Office of Continuing Medical Education, University of California, Davis, Medical Center, 2701 Stockton Boulevard, Sacramento, CA 95817. Telephone: (916) 734-5390; FAX: (916) 736-0188.

Second Consensus Development Conference on the Treatment of Radiation Injuries: Marrow Aplasia, Sepsis, Gastrointestinal Injury, and Radiation Accidents, April 14–17, 1993, Bethesda, MD. Contact: Dr. Doris Browne, Armed Forces Radiobiology Research Institute/MRA, 8901 Wisconsin Avenue, Bethesda, MD 20889-5603. Telephone: (301) 295-0316; FAX: (301) 295-5673.

Critical Issues in Tumor Microcirculation, Angiogenesis, and Metastasis: Biological Significance and Clinical Relevance—A Continuing Education Course, June 7–11, 1993, Harvard Medical School, Boston, MA. Credits: 22 hours Category 1 AMA. Contact: Dr. Norman Shostak, Department of Continuing Education, Harvard Medical School, 641 Huntington Avenue, Boston, MA 02115. Telephone: (617) 432-1525; FAX: (617) 432-1562.


Errata

Two errors have been found in the article by Toyokuni et al., entitled "Combined Histochemical and Biochemical Analysis of Sex Hormone Dependence of Ferric Nitritrolactate-induced Renal Lipid Peroxidation in ddY Mice," which appeared in the September 1, 1990 issue of Cancer Research (pp. 5574–5580). In Table 1, the column labeled "Estriol" under the "Female" heading should have been labeled "Testosterone." In addition, in the legend to Fig. 3, the word "patchy" should be omitted from the expression "diffuse patchy Schiff-positive areas."

Coauthor Timothy J. Cuzi's name was misspelled as Timothy J. Cuzi in the article by Leteurtre et al., titled "Rational Design and Molecular Effects of a New Topoisomerase II Inhibitor, Azatoxin," which appeared in the August 15, 1992 issue of Cancer Research (pp. 4478–4483).
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