Carcinogenesis vol.19 no.7 pp.1285–1290, 1998

Enhancement of estrogen-induced renal tumorigenesis in hamsters by dietary iron

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Iron participates in the generation of hydroxyl radicals by the iron-catalyzed Fenton reaction. Its role in estrogen-induced carcinogenesis has been examined in this study by investigating the effects of iron content of hamster diets on tumor induction by estradiol. The renal tumor incidence and number of tumor nodules in hamsters treated with estradiol plus a diet enriched with iron (384 p.p.m. Fe as ferric citrate) for 5 months were 2- and 4-fold higher, respectively, than those observed in animals on an iron-poor diet plus estradiol (3.9 p.p.m. Fe, as ferric citrate). Tumor incidence and number of tumor nodules in estradiol-treated hamsters on the iron-deficient diet were not different from those of animals on a normal rodent chow. No tumors were detected in hamsters treated only with the low or high iron diets. Total serum iron was significantly increased in animals treated with the high iron diet plus estradiol compared with the low iron diet plus estradiol group and the high and low iron controls. Estrogen treatment increased non-heme iron in liver of both high and low iron treatment groups and in kidney of the hamsters on the low iron diet. It is concluded that dietary iron enrichment enhances the incidence and severity of estrogen-induced tumor induction.

Introduction

The chronic administration of estradiol (E2*) to male Syrian hamsters for ~6–7 months induces renal tumors with 80–100% incidence (1). Free radicals generated by metabolic redox cycling of catecholestrogen metabolites have been postulated to play a role in this tumor induction by estrogens (2). Specifically, the redox cycling of 4-hydroxyestradiol between hydroquinone (the catechol) and quinone forms may be a source of reactive radicals, which induce potentially mutagenic DNA alterations (3,4). Consistent with this hypothesis, catecholestrogen-semiquinone intermediates have been shown to react with molecular oxygen and to produce superoxide *in vitro* (5,6). Moreover, DNA single strand breaks have been induced in MCF-7 breast cancer cells by estrone-3,4-quinone, presumably by reactive radicals generated by metabolic redox cycling (7).

In Syrian hamsters, free radical formation induced by estrogen prior to the development of kidney tumors is indicated by several types of cell damage, which illustrate the operation of metabolic redox cycling of catecholestrogens *in vivo*. For instance, the induction of DNA single strand breaks, of 8-hydroxylation of guanine bases of DNA and of lipid peroxidation in hamsters treated with E2 or 4-hydroxyestradiol all indicate the formation of hydroxyl radicals by this hormone and their deleterious action in the hamster (4,8,9). The conversion of the less reactive superoxide radicals, formed primarily by metabolic redox cycling of catecholestrogen metabolites, to the highly reactive hydroxyl radicals evident from the nature of the cellular damage induced by E2 *in vivo*, requires the reduction of hydrogen peroxide or lipid peroxides by metal ions, Cu^{+} or Fe^{2+}, in a Fenton reaction (10).

The source and role of metal ions, specifically iron, in estrogen-induced carcinogenesis has not received much attention and is the focus of this study. A diet moderately enriched with iron (384 p.p.m. Fe compared with 215 p.p.m. Fe in regular rodent chow) was expected to enhance the incidence of E2-induced kidney tumors in hamsters, if hydroxyl radical formation by an iron-mediated Fenton reaction was a necessary part of the carcinogenesis process. In contrast, a minimal-iron diet was expected to result in a lower tumor incidence. Because of the requirement of iron for many biological functions and processes of cells, a minimal-iron diet (3.9 p.p.m. Fe) had been chosen, which has been previously shown to support the growth and health of these animals (11). One group of E2-treated hamsters received normal rodent chow to permit a comparison of tumor incidence data with previously established values.

In addition to tumor incidence, we also examined several parameters of iron metabolism and distribution in animals that received the specially formulated high or low iron diets to permit an assessment of the fate of the administered iron and its role in the tumorigenesis process. For this purpose, we measured the total iron concentrations and the iron binding capacity in serum. In addition, non-heme iron concentrations were assayed in kidney, the target of estrogen-induced tumorigenesis, and for comparison purposes, in liver, where tumors do not develop by this treatment. Finally, the distribution of iron deposits was also examined in kidney and liver sections by Gomori staining. Such studies of iron metabolism and distribution were not carried out with hamsters on normal rodent chow, because this regular diet likely contains antioxidants and other modulators of tumor incidence and iron metabolism.

Material and methods

Materials

Chemicals. Ferrozine, bathophenanthroline sulfonate, neocuprein, sodium acetate, hydrochloric acid, ferrous ammonium sulfate, ascorbic acid, ammonium acetate, Fast Red and trichloroacetic acid were all purchased from Sigma (St Louis, MO). Buffered formalin and an atomic absorption iron standard (ferric nitrate) were purchased from Fisher (Pittsburgh, PA). All chemicals in this study were reagent grade or better.

Diets. Low iron (TD 96046: 3.9 p.p.m. Fe as Fe-citrate) and high iron (TD 96047: 384 p.p.m. Fe as Fe-citrate) diets were purchased from Harlan Teklad (Madison, WI). The diets were prepared by adding appropriate amounts of ferric citrate to a basal diet of defined composition (Table I). A normal rodent diet (Regular Lab Diet 5008-A: 215 p.p.m. Fe as ferrous carbonate) was purchased from PMI Feeds (St Louis, MO). The iron content of diets was

***Abbreviation:** E2, 17β-estradiol.

Table I. Composition of the basal diet used in this study. This basal diet was used as the low iron diet. An appropriate amount of ferric citrate was added to the basal diet to achieve the desired iron content of the excess iron diet

Ingredients	Mass (g/kg)
Casein	200.0
DL-methionine	3.0
Sucrose	332.6
Corn starch	332.0
Corn oil	50.0
Cellulose	25.0
Vitamin mix, Teklad (40060)	10.0
Potassium citrate, monohydrate	18.0
Calcium phosphate, dibasic[a]	15.9
Calcium carbonate[a]	4.4
Sodium chloride	3.7
Magnesium oxide	2.2
Manganous carbonate	0.1
Zinc carbonate	0.06
Chromium potassium sulfate	0.02
Cupric carbonate	0.014
Potassium iodate	0.001
Sodium selenate	0.0004

[a]Reagent grade compounds.

determined by dry ashing 2 g of feed overnight at 550°C in a Fisher ISOTEMP muffled furnace, Model 186A, followed by dissolving the ash in 3 *N* HCl. After adding 0.36 *N* HCl to obtain a defined sample volume, atomic absorption analysis was carried out using a Perkin–Elmer atomic absorption spectrophotometer, Model 2380. A standard curve was prepared from the absorption of standard solutions of ferric nitrate and their iron contents calculated.

Tumor induction

Male Syrian hamsters, 6–8 weeks old, were purchased from Harlan/Sprague–Dawley (Houston, TX). All animals were housed in plastic cages and acclimatized for 1 week. Hamsters were randomly separated into groups with an average body wt of ~86 g, within a group, and then received either a low iron diet (3.9 p.p.m. iron as Fe-citrate), a low iron diet plus E2, a high iron diet (384 p.p.m. iron as Fe-citrate), a high iron diet plus E2, or a normal rodent diet plus E2. The animals received their respective diets and drinking water in glass bottles *ad libitum*. The iron content of the water was below the detection limit of the atomic absorption method used (0.003 mg Fe/liter). After 1 week of acclimatization, estrogen treatment was started by subcutaneous implantation of E2 (one 25 mg E2 pellet containing 10% cholesterol) as previously described (12). The average body wt of each group of animals was recorded monthly to monitor any effect the diet might have on the health of the animals. After 3 months, estrogen-treated hamsters received an additional E2 pellet to ensure a continuous supply of estrogen, as described previously (12). After 5 months, all hamsters were killed by decapitation. Blood was collected, and animals were visually inspected for any metastases. Renal tumor nodules were counted to determine the tumor incidence. Large tumors (>3 mm in diameter) were listed separately from smaller tumor nodules. Portions of kidney and liver tissue were placed on dry ice and kept frozen at –80°C for further studies. Other portions were placed in 10% buffered formalin for microscopic analyses of the kidney tumors and for histochemical localization of iron by Gomori's staining (13).

Serum iron and total iron-binding capacity

Total serum iron was assayed according to the method of Carter (14). A mixture of 0.25 ml serum and 0.25 ml reducing agent (0.02% ascorbic acid in 0.2 *N* hydrochloric acid) in disposable polyethylene tubes was allowed to stand at room temperature for 5 min. To this mixture, 0.25 ml of aqueous trichloroacetic acid (11.3%) was added. The tubes were capped to prevent evaporation and then centrifuged at 2000 *g* for 20 min. An aliquot (0.50 ml) of the clear supernatant was transferred to another tube and mixed with 0.4 ml of 10% ammonium acetate buffer and 0.1 ml ferrozine solution (prepared from 75 mg ferrozine and 75 mg neocuprein in 25 ml distilled water, then acidified with one drop of concentrated hydrochloric acid). This mixture was incubated for 5 min at room temperature and assayed spectrophotometrically at 560 nm for the formation of Fe(II)-ferrozine complex. A standard curve prepared with standard solutions of ferrous ammonium sulfate was used to calculate total serum iron.

Total iron-binding capacity was assayed using Carter's (14) method for total serum iron as described above, with one modification. Instead of adding 0.25 ml reducing agent to the serum sample, 0.25 ml of a ferrous ammonium sulfate solution (final concentration of 5.7 mg Fe/l). was added to saturate the serum transferrin completely. Then the saturated transferrin was precipitated and the supernatant used to measure the excess unbound iron. The excess unbound iron was subtracted from the total saturating iron added, and the difference added to the total serum iron (determined in parallel) to give the total iron-binding capacity.

Non-heme iron

Non-heme iron was determined by the method of Torrance and Bothwell (15). Weighed sections of kidneys and livers in mixtures of equal volumes of 6 *N* hydrochloric acid and 20% trichloroacetic acid were placed in a GS Blue M electric oven, model SM 11TA-1 at 65°C for 20 h. After digestion, samples were allowed to cool to room temperature. Aliquots of the yellow supernatants (0.2 ml) were mixed with 2.8 ml of bathophenanthroline-color reagent (0.56 mg bathophenanthroline sulfonic acid in 25 ml of saturated aqueous sodium acetate and 25 ml of distilled water) and incubated at room temperature for 10 min. Finally, the absorption of the bathophenanthroline-Fe(II) complex was determined spectrophotometrically at 530 nm, and the concentration of tissue non-heme iron was assayed using standard solutions containing ferrous chloride.

Histological and histochemical analyses

Kidney and liver slices in buffered formalin were stored at 4°C until histological and histochemical analyses were performed. Tissues were embedded in paraffin for the preparation of 5 μm sections. Sections were stained according to Gomori's procedure for Fe^{3+}, as described by Sheehan and Hrapchak (13) and counterstained with Fast Red. Sections were microscopically examined to determine deposits of ferritin and the iron pigment hemosiderin formed by denaturation of ferritin loaded with an excess of iron (16). All histological and histochemical analyses were carried out using an Olympus microscope, model AH-2, purchased from Dexter Instrument (San Antonio, TX).

Statistical analysis

Means of body wt, total serum iron levels, total iron binding capacity and tissue non-heme iron concentrations were analyzed by one-way ANOVA. Parameters with $P < 0.05$ obtained by ANOVA analyses were further analyzed for differences between groups by the Tukey's or Student–Newman–Keuls' tests. Tumor incidence was analyzed by Fisher's exact test (one-tailed). Tumor burden and tumor growth were analyzed by chi-squared test. All statistical analyses were performed using SigmaStat 2.0 software, except for the analysis of tumor incidence, which was carried out using a GraphPAD Instat 1.12a program from GraphPAD Software.

Results

Effects of iron and estrogen on body weight

There was no significant difference in average body weight of estrogen-treated or control hamsters after consumption for 5 months of a low iron (3.9 p.p.m. Fe) diet or a diet 78.6% higher in iron (384 p.p.m. Fe) than normal rodent chow (215 p.p.m. Fe) (one-way ANOVA, lowest *P*-value = 0.31) (Figure 1). The average body weights of hamsters treated with E2 and the low iron diet for 2–5 months appear lower (152–159 g) than the body weight ranges observed with the other groups (170–184 g) in that time period. However, large variations within the E2 plus low iron diet group (130–209 g) indicated that at the 5 month time period, reasons other than diet are potentially responsible for the lower average body weights of the E2-treated hamsters on the low iron diet. The comparable rate of body weight increase during the initial growth phase also supports this conclusion.

Effect of iron on estrogen-induced carcinogenesis

Estrogen-treated hamsters on a normal rodent chow for 5 months had a tumor incidence of 50% (Table II). This tumor incidence is in line with previous observations of a 100% incidence by exposure to estrogen for 6–8 months and lower tumor incidence after shorter treatment periods (1,12). No differences were detected in tumor incidence and renal tumor burden between these hamsters on a normal rodent chow and

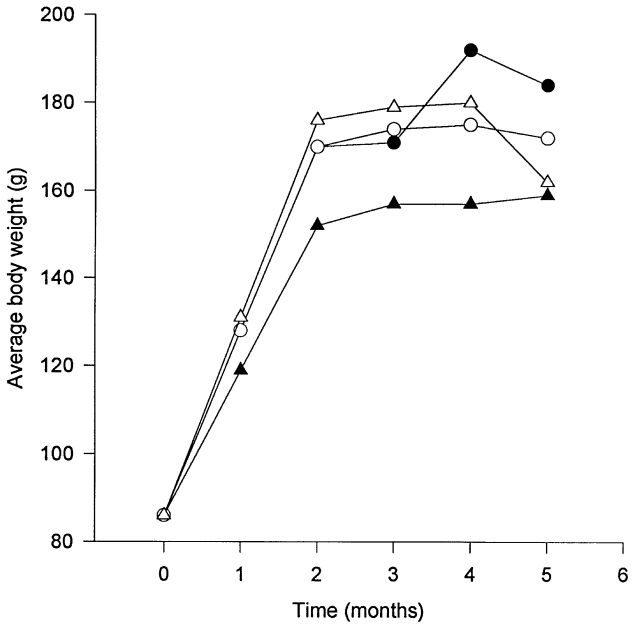


Fig. 1. Effects of diets and/or estrogen treatment on body wts of hamsters. Animals received a diet containing 3.9 p.p.m. Fe (●), 384 p.p.m. Fe (○) or the low or high iron diet in addition to E2 treatment (▲ or △, respectively). Average body wts of hamsters were obtained at indicated intervals and expressed as means ± SD. Errors bars were omitted for the sake of clarity.

Table II. Influence of modified iron diets on renal carcinogenesis induced in hamsters by E2 treatment for 5 months

Treatment	No. of animals with tumors/ no. of animals (%)	Mean no. of tumor nodules/hamster	Mean no. of large tumors (>3 mm)/hamster
Low iron	0/10 (0)	0	0
High iron	0/12 (0)	0	0
Normal iron + E2	5/10 (50)[a]	2.40 ± 0.86[b]	0.20 ± 0.19
Low iron + E2	6/15 (40)[a]	3.47 ± 1.59[b]	0.07 ± 0.06
High iron + E2	11/14 (79)	14.71 ± 4.42	0.57 ± 0.27

Male Syrian hamsters treated with E2 implants or controls were given low iron (3.9 p.p.m. Fe) or high iron (384 p.p.m. Fe) diets for 5 months. An additional group of hamsters received E2 implants and a normal rodent chow (215 p.p.m. Fe). The animals were then killed and their kidneys were macroscopically examined for the number of large (>3 mm) and small (<3 mm) renal tumor nodules on both kidneys. The macroscopic analysis was confirmed by histological examination of hematoxylin and eosin stained sections.
[a] $P < 0.05$ compared with high iron diet + E2-treated animals by Fisher's exact (one-tailed) test.
[b] $P < 0.05$ compared with high iron diet + E2-treated animals by chi-squared test.

animals on a low iron diet. In contrast, the high iron diet doubled the estrogen-induced tumor incidence over that in animals on a low iron diet [$P < 0.05$ by the Fisher exact (one-tailed) and chi-square tests]. The high iron diet also significantly increased the mean number of tumor nodules/hamster by >4-fold compared with the low iron diet + E2 group ($P < 0.05$) (Table II). There was no significant difference in the number of large tumor nodules between these latter two groups that bore tumors (Table II). A low iron diet or a high iron diet consumed by control hamsters for 5 months did not induce any renal tumors.

Table III. Total serum iron and total iron binding capacity in hamsters treated with E2 and/or low iron and high iron diets for 5 months

Treatment	Total iron (μg/dl)	Total iron-binding capacity (μg/dl)
Low iron	222.39 ± 17.94[a]	688.45 ± 43.94
High iron	212.94 ± 22.42[a]	726.82 ± 38.89
Low iron + E2	200.42 ± 46.42[a]	584.67 ± 61.68[b]
High iron + E2	280.97 ± 22.24	599.34 ± 33.07[b]

Male Syrian hamsters treated with E2 implants or untreated controls were given low iron (3.9 p.p.m. Fe) or high iron (384 p.p.m. Fe) diets for 5 months. The animals were then decapitated and their blood was collected. Total serum iron was assayed by the formation of Fe-ferrozine complex by the method of Carter (14). Total iron-binding capacity was determined using a modification of Carter's (14) method for total serum iron, as described in Materials and methods. Values are means ± SD ($n = 5$ for both measurements).
[a] $P < 0.027$, significantly different from high iron diet + E2 (one-way ANOVA followed by Student–Newman–Keuls test).
[b] $P < 0.006$, significantly different from high iron diet and low iron diet control groups (one-way ANOVA followed by Tukey's test).

Table IV. Non-heme iron in kidneys and livers of hamsters chronically treated with E2 and/or low and high iron diets plus E2 for 5 months

Treatment	Kidney (μg Fe/g wet wt)	Liver (μg Fe/g wet wt)
Low iron	351.74 ± 92.01[a]	544.22 ± 79.04[b,c]
High iron	379.05 ± 36.02[a]	494.87 ± 76.36[b,c]
Low iron + E2	669.69 ± 66.09	713.46 ± 28.04
High iron + E2	346.39 ± 83.93[a]	778.71 ± 72.12

Male Syrian hamsters were chronically treated with E2 and/or low iron (3.9 p.p.m. Fe) and high iron (384 p.p.m. Fe) diets for 5 months and were then killed by decapitation. Portions of the kidneys and livers were kept at –80 °C until analyzed for non-heme iron according to the method of Torrance and Bothwell (15) as described in Materials and methods. Values are means ± SD ($n = 3$–4 for either kidneys or livers).
[a] $P < 0.001$, significantly different from non-heme iron in kidneys of low iron diet + E2-treated group (one-way ANOVA followed by Tukey's test).
[b] $P < 0.001$, significantly different from non-heme iron in livers of low iron diet + E2-treated group (one-way ANOVA followed by Tukey's test).
[c] $P < 0.001$, significantly different from non-heme iron in kidneys of high iron diet + E2-treated group (one-way ANOVA followed by Tukey's test).

Total serum iron and total iron binding capacity

The total serum iron concentration in the high iron + E2 group was significantly higher than in the low iron + E2 group (41%) and also higher compared with the low iron and high iron controls, 26 and 31%, respectively ($P < 0.027$) (Table III). In contrast, the different iron concentrations in the diets did not affect total serum iron concentrations in the absence of estrogen exposure. Moreover, there was no difference in values between serum iron levels in these control hamsters and those in the low iron + E2 group (Table III). E2 exposure significantly decreased the total iron binding capacity in serum of hamsters treated with the low iron and high iron diets compared with their respective controls by 12 and 10%, respectively ($P < 0.006$, one-way ANOVA followed by Tukey's test) (Table III). The variation of iron concentrations in the diet did not affect total iron binding capacities in hamsters lacking E2 exposure.

Tissue non-heme iron

Non-heme iron levels in the kidneys of E2-treated hamsters on the low iron diet were significantly higher than corresponding levels in animals on the high iron diet (94%) and also

higher than those in control hamsters on high iron and low iron diets (90 and 77%, respectively, $P < 0.001$) (Table IV). In contrast, the concentrations of tissue non-heme iron in control animals not exposed to estrogen were not affected by the differential iron levels in the diets. Estrogen treatment of animals consuming low or high iron diets significantly raised hepatic concentrations of non-heme iron compared with their respective controls (31 and 44% respectively, $P < 0.001$) (Table IV). Hepatic non-heme iron concentrations were also higher than those in kidney that was independent of the dietary iron levels. As was observed in the kidney, the non-heme iron concentrations of liver in animals lacking E2 exposure were not affected by the differential iron levels in the diets.

Localization of iron deposits

There were no iron deposits identifiable by Gomori stain in kidney and liver of hamsters treated with E2 and a low iron diet, or in low iron diet controls (Figure 2A–D, respectively). In contrast, stainable iron accumulated in some hepatocytes of hamsters receiving the high iron diet (Figure 2E). This iron uptake was further enhanced by estrogen treatment in liver cells of hamsters on a high iron diet (Figure 2F). In the kidneys of these animals, small isolated deposits of stainable iron were occasionally detected in renal epithelial cells (Figure 2G). In the kidney tumors of these animals, nests of tumor cells with high amounts of iron deposits were detected in many tumors (Figure 2H). Kidney sections of hamsters treated with only the high iron diet did not contain stainable iron (data not shown).

Discussion

Our data establish a co-carcinogenic role of iron in estrogen-induced carcinogenesis. An elevated supply of iron in the diet increased both the incidence of kidney tumors induced by estrogen and the severity as expressed in the number of tumor foci per hamster. The iron enrichment in the diet needed for this enhancement of tumor incidence was <80% above levels in normal rodent chow. Our data are consistent with previous reports of an enhancement by elevated dietary iron of mammary carcinogenesis induced by dimethylbenz[*a*]anthracene or *N*-methylnitrosourea (11,17–19). They are also consistent with tumor inhibition by iron deficiency as reported previously (11,18,20).

The hamsters on the low iron diet thrived, and their body weights after 5 months of treatment were not significantly different from those of the other animals. However, it is possible that these animals suffered from iron deficiency. The blood hemoglobin and hematocrit values in rats on a diet with a higher iron content (5 or 8 mg Fe/kg diet) than used in hamsters in our study (3.9 mg Fe/kg diet) were reduced by 50 and 40%, respectively, from control levels as reported by Hrabinski *et al*. (18) and Spear and Sherman (20). Our measurements of iron status did not suggest any anemia in estrogen-treated or control hamsters on the low iron diet. However, it is possible that these animals were partially iron deficient, which needs to be investigated further with additional parameters of iron status in future studies.

Our data clearly demonstrate that estrogen treatment of hamster resulted in tissue accumulation of non-heme iron in the liver and also in the kidney (at least in animals on a low iron diet). This concept is also supported by histochemical analyses. Iron deposits were detected by Gomori staining occasionally in renal tubular cells of hamsters treated with E2 plus the high iron diet, and at higher concentrations in kidney tumors. High iron levels were also deposited in the livers of these animals. The renal non-heme iron concentrations in these estrogen-treated animals on a high iron diet were not elevated, but their serum iron concentration and total iron binding capacity differed from those of controls. It is possible that in kidneys with a large tumor burden, iron was mobilized from storage in kidney and placed in circulation. It is also possible that iron levels are lower in kidneys that contain many rapidly proliferating tumors than kidneys with a lower tumor burden. These possibilities require further study in future investigations. The tissue uptake of iron induced by estrogen is independent of the dietary status and is observed even in hamsters on an iron-deficient diet, which clearly demonstrates the role of estrogenic hormones in the regulation of iron metabolism. Our data are consistent with the previously reported increase by E2 of serum iron concentrations (21,22) and the greater iron stores in kidneys and livers of female compared with male rats (23–25). The reason for this accumulation of iron may be the induction by estrogen of transferrin receptors (26). This or other possibilities require further study.

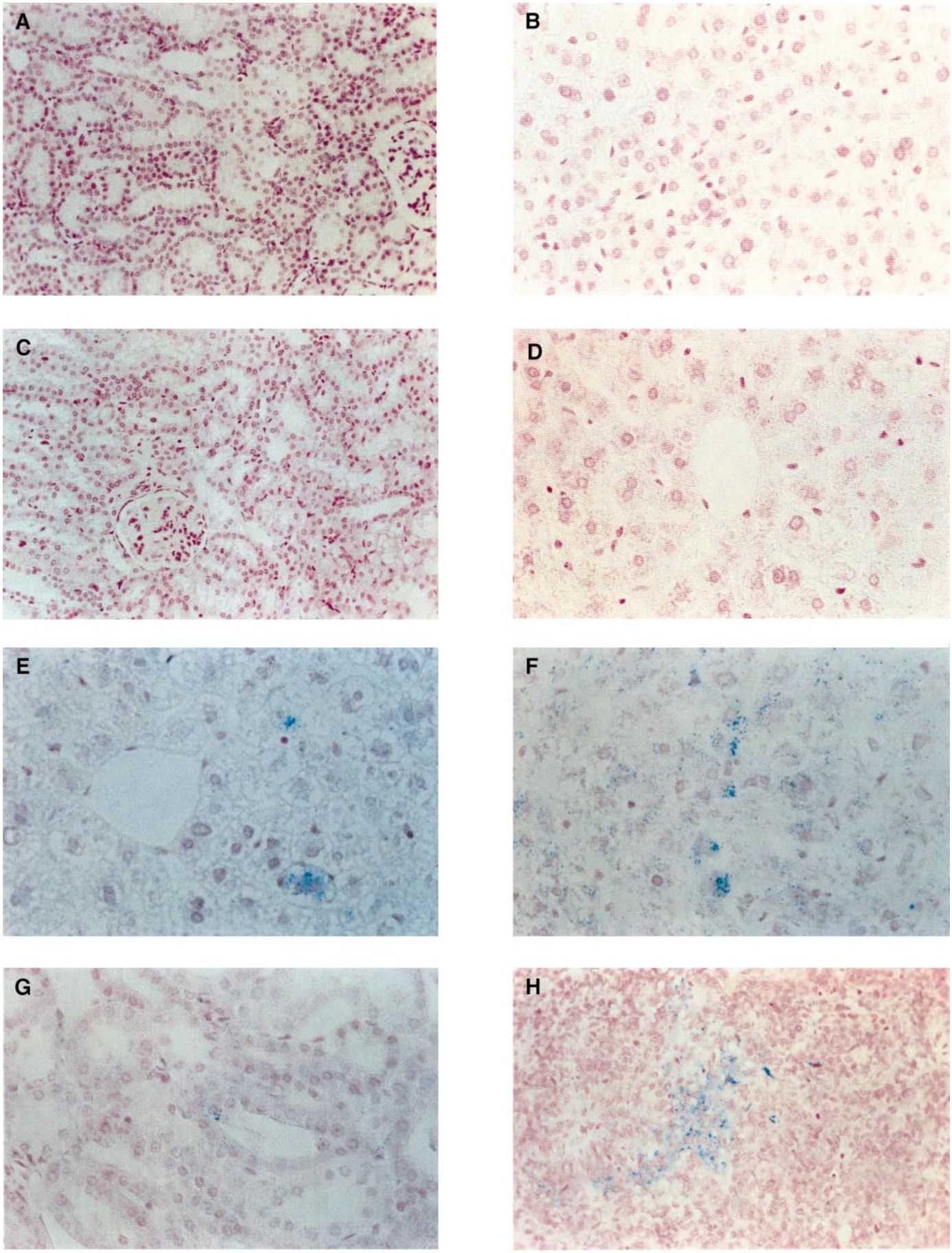
The iron deposits in kidney tumor and, to a lesser extent, in normal kidney cells indicate that iron accumulation may participate in the estrogen-induced tumorigenesis process in cells with high iron stores, or in their vicinity. Iron may enhance tumorigenesis in renal cells already sensitive to hormonal carcinogenesis by inducing iron-mediated free radicals. Hepatic cells also accumulate iron and contain iron deposits as illustrated by Gomori staining, and possibly may be damaged by this iron. However, tumors may not develop in this organ as hamster liver is markedly less sensitive to estrogen-induced carcinogenesis than hamster kidney.

Our data support a mechanism of tumor induction by free radicals generated by redox cycling of 4-hydroxylated catecholestrogens as postulated previously (2,27). The accumulated iron in kidney may participate in hydroxyl radical production via the Fenton reaction and increase the estrogen-induced DNA damage by free radicals, as described previously (2,27). Tumors may develop from such damaged cells, which may respond to proliferative stimuli via estrogen receptor-mediated processes. The enhancement of estrogen-induced tumorigenesis by iron is not consistent with a mechanism of tumor induction based solely on receptor-mediated proliferative processes, as postulated previously (28).

Acknowledgements

The authors would like to thank Dr Edward Posthlewait of the University of Texas Medical Branch (Galveston, TX) for his suggestions and discussions, Dr Ron Rose of Harlan Teklad (Madison, WI) and Dr Henry Thompson of AMC Cancer Research Center, University of Colorado (Denver, CO) for their

Fig. 2. Gomori staining of kidney and liver sections of hamsters treated with E2 and/or low or high iron diets for 5 months. (**A** and **B**) Kidney and liver sections of a hamster treated with a low iron diet plus E2 (total magnifications of ×62.5 and ×125, respectively). (**C** and **D**) Kidney and liver sections, respectively, of a hamster treated with a low iron diet (total magnifications of ×62.5 and ×125, respectively). (**E**) Liver section of a hamster treated with the high iron diet. Please note the iron deposits stained dark blue (total magnification of ×125). (**F**, **G** and **H**) Liver, kidney and tumor sections of a hamster treated with high iron + E2. Please note the iron deposits stained dark blue (total magnifications of ×125, ×125 and ×62.5, respectively).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recommendations concerning diet composition, and Pat Callahan for his assistance with the microscope and preparation of the photomicrographs. This work was supported by a grant from the National Institutes of Health, NCI (CA 74971). S.W. is enrolled in a Toxicology Training Program (NIEHS T32-ES-07254). J.G.L. is an NIEHS Center grant investigator (ES06676).

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Received on January 5, 1998; revised on March 26, 1998; accepted on April 9, 1998