Introduction

Iron is an essential element, but too much and too little of it may have adverse health consequences. Several epidemiological studies suggest that an excess of body iron stores could increase the risk of cancer (1–4). Iron overload can result from ingestion, inhalation, injection or a pathophysiologic process (5–8). Iron may increase the risk at some cancer sites and not others (7,9). A positive association has been observed between body iron stores and cancers of the liver, lung and intestine, while cancer of the stomach appeared to be unrelated to iron status (reviewed in 1). The tumors are often associated with the sites of deposition of the metal.

Experimental evidence suggests that animals with high body iron stores tend to be at greater risk than normal animals in the development of neoplasms. Evidence exists associating iron with both initiating and promoting phases of carcinogenesis. Thus, intramuscular injections of large quantities of iron induce injection site sarcomas (6). The incidence of neoplasms in the lungs of hamsters, which had inhaled diethylnitrosamine, were shown to increase significantly with concomitant inhalation of iron oxide, suggesting its co-carcinogenic effects (5). Iron given in the diet during the initiation phase was shown to enhance 1,2-dimethylhydrazine-induced colon carcinogenesis in mice (10). Furthermore, a second study presented evidence that dietary iron administered during the promotion process also increased both tumor yield and the incidence of colon cancer (11).

In recent studies, the hypothesis of body iron-stores and the risk of cancer was extended to another important target organ, the breast. Thus, dietary iron appeared to enhance the rate of appearance of mammary tumors initiated by N-nitrosomethyl-urea (NMU*; 12,13). However, in those studies, differences in tumor incidence, tumor multiplicity and cancer latency compared with the control group were not significant by statistical test. A high dose of NMU given to animals in that study gave rise to a >50% incidence of tumors in the controls, thus weakening the sensitivity of the experiment. The present study is based on the standard Sprague–Dawley (SD) female mammary tumor model (Huggins’ model, 14) using dimethylbenz[a]anthracene (DMBA) as the carcinogen, but at a low dose of DMBA (5 mg/kg body wt) given at 55 days of age to minimize the yield of tumors in the control group.

Materials and methods

Animals

Female SD rats were purchased from Taconic Farms (Germantown, NY) at 48 days of age. Animals were housed three to a polycarbonate cage on hardwood chip bedding and fed NIH-31 diet (Zeigler Brothers, Gardiners, PA) and water ad libitum. The NIH-31 diet contains 345 p.p.m. iron in the form of ferrous sulfate. The animals were housed at a temperature of 68–72 °F and a relative humidity of 50 ± 5%, with a 12-h light–dark cycle. Animal care was provided in accordance with the procedures outlined in the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 86-23, 1985).

Treatment

The experimental design used in the present study is shown in Figure 1. Specifically, rats were randomized into four groups of 30 each. At 55 days of age, rats in experimental groups (groups 1 and 2) received i.g. administration of 5 mg DMBA/kg body wt in olive oil. Controls (group 3) received equivalent volumes of olive oil only. Eight days later, animals in groups 2 and 3 received s.c. injections of iron (ferrous sulfate; 50 µmol/kg) twice a week for 53 weeks. Ferrous sulfate was dissolved in argon purged saline and administered s.c. to facilitate complete absorption of the administered dose. Group 4 was an untreated control group. The size and location of the tumor masses were recorded every week starting from the first appearance of tumor. Sizes were recorded as diameter of the widest part of the tumor.

Necropsy and histopathology

Dead and moribund rats, and rats with tumor masses >5 cm, were necropsied. All remaining animals were killed at 62 weeks of age (i.e. 54 weeks after initiation). Body weights were recorded and all mammary masses and normal mammary glands were removed and weighed. Portions of mammary masses and spleens were frozen in liquid nitrogen for future molecular studies. Remaining portions of mammary masses, and all other tissues with lesions, were fixed in 10% neutral buffered formalin, trimmed and embedded in paraffin. Mammary tumors were classified as fibroadenomas, adenomas and

*Abbreviations: DMBA, 7,12-dimethylbenz[a]anthracene; NMU, N-nitrosomethylurea; SD, Sprague–Dawley; H&E, hematoxlin and eosin.
adenocarcinomas using the criteria described by Young and Hallowes (15). Tissue sections were stained with hematoxin and eosin (H&E). To detect ferric ions, selected sections of normal, hyperplastic and malignant mammary tissues were subjected to Gomori’s modified iron/Prussian blue reaction (16).

Statistical analysis
Fisher’s exact test was used in pair-wise comparison of incidence of mammary tumors. Data on body weight gain, tumor size and multiplicity (number of tumors/tumor-bearing rat) of tumors were examined by Dunnet’s t-test and Kruskal–Wallis non-parametric ANOVA test. In all cases, a probability of $P < 0.05$ was considered to indicate a significant difference.

Results

Body weight gain and survival
Body weight gains were not significantly different between different groups of rats (Figure 2). However, from week 30 to week 50, body weight gain in the DMBA/iron group was slightly but consistently lower than that in the saline-treated group.

Survival was moderately affected in rats exposed to iron following DMBA initiation (Figure 3). A significant difference in the survival rate between the DMBA/iron group and two control (iron-only and untreated) groups was observed after week 40 ($P = 0.03$). Survival was also significantly affected in the DMBA-only group after week 50 ($P = 0.03$ compared with control groups). Although the survival in the DMBA/iron group was more severely affected than in the DMBA-only group, the difference was not of statistical significance.

Mammary tumors
Mammary tumors started to appear 7–10 weeks after DMBA initiation. The first such tumor was seen in the DMBA/iron sulfate group at 109 days of age (i.e. ~6.5 weeks following DMBA administration). Although the tumor incidence increased thereafter in both experimental groups, the increase was more pronounced in the iron-treated animals than in the DMBA-only group (Figure 4). Thus, at 35 weeks after treatment with DMBA, the mammary tumor incidence was significantly increased.
higher \( (P = 0.001) \) in the DMBA/iron group than in the DMBA-only group. The tumor incidence in the DMBA/iron group increased further as the promoter treatment continued and reached almost 90% at week 50 following DMBA initiation \( (P = 0.0001) \) as compared with the DMBA-only group). A small number of rats (4/30; 13%) in the iron-only group also developed mammary tumors (Figure 4), while such tumors occurred in only one untreated control rat. The incidence of mammary tumors in the DMBA-only group was significantly higher than in the iron-only group after ~30 weeks of promoter treatment. Injection site sarcomas, found in two rats given iron only group and one rat in the DMBA/iron group, were not included in Figure 4.

Although the size of mammary tumors increased over time for DMBA-only rats between 20 and 50 weeks (9.0 ± 1.9 versus 22.2 ± 3.6; \( P = 0.03 \)) and DMBA/iron rats between 10 and 50 weeks (10.0 ± 2.9 versus 24.7 ± 2.06; \( P = 0.01 \)), the mean diameters of these tumors in DMBA/iron rats were consistently larger than in DMBA-only rats (Figure 5). Thus, mammary tumors in DMBA/iron rats were 2.3 times \( (P = 0.04) \) larger than those in DMBA-only rats at 20 weeks and 2.0 times larger at 30 weeks \( (P = 0.05) \) following DMBA initiation. After 40 weeks, the difference in the size of such tumors between DMBA/iron and DMBA-only groups was not statistically significant.

Multiple mammary tumors occurred mostly in DMBA-initiated animals. No significant differences were seen in the multiplicity of mammary tumors between DMBA-only and DMBA/iron groups at any observation time point (data not shown). However, at 53 weeks (i.e. at 62 weeks of age) when the experiment was terminated, the multiplicity of such tumors was greater in the DMBA-only and the DMBA/iron groups as compared with the iron-only group, a difference close to statistical significance \( (P = 0.07; \) Figure 6).

Mammary tumors in the DMBA-only and DMBA/iron groups were mostly adenomas and adenocarcinomas (85–88%;...
in mammary adenocarcinomas induced by DMBA alone. It should be noted here that this histochemical method may not detect all forms of bound iron and some undetected iron may be present in the neoplastic cells. Iron was, however, easily detectable by this method in the stromal tissue and hyperplastic mammary glands initiated by DMBA and promoted by iron (Figure 8B). As shown earlier by Singh et al. (13), epithelial cells from benign mammary tumors (adenomas) were positive for iron (Figure 8C) but malignant epithelial cells were devoid of such ions (Figure 8D). However, iron was detectable in the stromal tissue adjacent to adenocarcinomas.

Discussion

The results of our study clearly show that iron administered s.c. as ferrous sulfate exhibits a strong promoting activity for mammary tissue in female SD rats. This promoting effect is evident from the fact that the administration of this metal following DMBA initiation greatly shortened the latency period for mammary tumor appearance and significantly increased their incidence as compared with rats not receiving such treatment. Also, the size of mammary tumors in iron-promoted rats was consistently larger than in DMBA-only rats until the final observation time point at 54 weeks after initiation. The occurrence of a small incidence of mammary tumors in non-initiated rats in this study is not totally surprising since the feeding of other established rat tumor promoters, phenobarbital alone or sodium barbital alone, similarly elicits a low incidence of liver and/or renal tumors in rats (17,18).

Iron was not detectable histochemically in normal mammary glands of iron-treated animals. It could be detected in both DMBA-initiated and spontaneous hyperplastic mammary tissue suggesting an altered physiologic state. Iron was also detectable in the epithelial cells of benign tumors, i.e. fibroadenomas and adenomas (Figure 8C). The significance of such focal accumulation of iron in the nature of its transportation (as well as binding) to preneoplastic mammary tissues is not yet clear (1,19).

Interestingly, however, iron was not detectable in the epithelial cells of mammary adenocarcinomas (Figure 8D) initiated by DMBA and promoted by iron. In such malignant tumors iron was found in stromal tissue as well as hyperplastic glands. Accumulation of iron in hyperplastic glands and stromal tissues surrounding malignant mammary glands as reported here, agrees with earlier findings by Vijayaraghavan and Rivera (19) and Sing et al. (13) who used an alternative route for iron administration. Such alterations in iron accumulation in preneoplastic versus neoplastic mammary tissues are in contrast to the observations made in liver. For example, hyperplastic nodules and tumors (adenomas and carcinomas), induced in rats by a variety of hepatocarcinogens, failed to accumulate iron when the surrounding liver was made siderotic by dietary administration of 8-hydroxyquinoline (20,21). Thus, the alterations in iron sequestration observed during the progression from normal to hyperplastic and benign lesions appear to depend upon the cell types altered.

Several mechanisms that may account for the effect of excessive iron on carcinogenesis have been proposed. Two lines of evidence provide biological rationale. First, iron may be a limiting nutrient for the growth and development of cancer cells (22,23): excess iron may increase the chances that cancer cells will survive and flourish (1). The histological findings of mammary glands and mammary tumors reported here, as well as in earlier studies (13), indicated significant differences in iron distribution within these tissues during mammary carcinogenesis. Of particular interest was the total absence of iron deposits in the malignant epithelial cells and its preferential accumulation in the stroma surrounding the adenocarcinomas. Similar findings were reported in Hodgkin’s disease by Smithyman et al. (24) who observed heavy deposits of ferritin and iron at the periphery of the tumor nodules. It is suggested that the accumulation of iron around the neoplasms possibly could be induced by the tumor cells to ensure a supply of this growth essential metal (1). This high iron requirement by the neoplastic cells may be related to their rapid rate of proliferation and high levels of transferrin-binding glycoproteins on their cell surfaces (25–27). Thus, transformed lymphoid cell lines have as many as 1000 times the number of transferrin receptors found on normal resting lymphocytes (28). It would be interesting to determine whether such differences in the number of receptors occur between the normal, preneoplastic and neoplastic mammary cells in rats exposed to iron following DMBA treatment. Also, since some siderophores (low molecular weight peptides involved in iron transport) have been shown to inhibit iron uptake from transferrin and produce anti-proliferative (and anti-tumor) effects in several in vitro and in vivo systems (29–31), it may be worthwhile to initiate further studies to examine the effects of such chelators on iron uptake and tumor development in the rat mammary gland.

Second, iron can catalyze the induction of oxygen radicals. In all aerobic organisms, iron as a divalent cation plays an important role in a series of reactions referred to as the Fenton Haber–Weiss cycle. Products of these reactions (i.e. hydroxyl radicals and other oxidants) have been shown to cause DNA strand breaks and mutagenicity (32,33). It is now widely believed that many tumor promoters commonly exert their effect in part through the accumulation of free radicals (34,35). Hydroxyl radicals also deplete intracellular stores of anti-carcinogens including anti-oxidant enzymes and low molecular weight scavenger molecules (36). Thus, preferential accumulation of iron in hyperplastic mammary glands and the subsequent
generation of the oxygen radicals may produce inflammation and hyperplasia in this tissue resulting in clonal expansion and selection (i.e. promotion) of carcinogen-initiated cells. Furthermore, oxy radical-induced damage to DNA and chromo-somes may also contribute to this process (35). This hypothesis is currently under investigation in our laboratory. Although Western diets include red meat, as well as widely used vitamin supplements, are very rich in iron (37,38), as far as we know there are very few epidemiological studies relating iron to breast cancer (39–41). Breast cancer is the most common cancer among women in the United States and the risk for this disease has been associated with dietary fat intake and inversely with dietary fiber (41,42). Nonetheless, many case control studies of fat consumption and breast cancer have found no clear or consistent relationship with either total fat, saturated fat or vegetable fat (43,44). Similarly, the relationship between total dietary fiber intake and subsequent breast cancer risk was very close to null in two recent prospective cohort studies (45,46). In light of these findings, epidemiological studies should examine more closely other dietary factors that may account for geographical variations in the risk for breast cancer. The results presented here and those reported by other investigators (12,13,19) strongly support such an argument.

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