Inhibitory effect of dietary iron deficiency on inductions of putative preneoplastic lesions as well as 8-hydroxydeoxyguanosine in DNA and lipid peroxidation in the livers of rats caused by exposure to a choline-deficient L-amino acid defined diet

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Effects of dietary iron deficiency on inductions of putative preneoplastic lesions and oxidative alterations in the livers of rats by a choline-deficient L-amino acid defined (CDAA) diet were examined. Male Fischer 344 rats, 4 weeks old, were used with a total experimental period of 16 weeks, consisting of 4-week pretreatment and 12-week treatment periods (periods A and B respectively). During period A, a choline-supplemented L-amino acid defined (CSAA) or an iron-deficient CSAA diet was administered, and the CDAA or an iron-deficient CDAA diet was fed in period B. Formation of 8-hydroxydeoxyguanosine (8OHdG), a DNA adduct generated by activated oxygen species, in DNA and lipid peroxidation in liver cell membranes were sequentially determined after the beginning of period B. At the end of the experiment, development of gamma-glutamyltransferase (GGT) and glutathione S-transferase placental form (GSTP) positive liver lesions were quantitatively analysed. In the animals fed the CDAA diet, formation of 8OHdG and lipid peroxidation increased with time, and GGT and GSTP positive liver lesions developed. Formation of 8OHdG, lipid peroxidation and the numbers of induced enzyme-altered liver lesions were all reduced in rats fed the iron-deficient CSAA diet in period A and/or the iron-deficient CDAA diet in period B. The present results indicate that iron plays an important role in induction of preneoplastic liver lesions in rats caused by exposure to the CDAA diet possibly in connection with its known catalytic role in generation of highly reactive activated oxygen species.

Introduction

Since Copeland and Salmon (1) first demonstrated that prolonged exposure to a diet low in choline and methionine induces neoplasms in the livers of rats, the underlying mechanisms of dietary choline (or methyl donors) deficiency have attracted a great deal of interest. It is now clear that a diet deficient in choline (or methyl donors) can itself result in hepatocellular carcinoma development without the additional presence of any known carcinogens (2—5). Strong promoting ability has also been observed (2,4,6—9). The essential causes of carcinogenicity, however, have not been clarified although roles for several events such as liver cell necrosis in association with subsequent regeneration (5,6,10), hypomethylation of DNA or RNA (11—14) and amplification of the c-myc gene (15) have been proposed. The possible contribution of oxy radicals in the carcinogenic processes including the stages of initiation, promotion and progression, has been paid particular attention (16—19). Also with regard to rat hepatocarcinogenesis induced by dietary choline (or methyl donors) deficiency, induction of DNA lesions and lipid peroxidation of the inner-nuclear, microsomal and mitochondrial membranes have been demonstrated in the livers of rats (4, 20—25). Recently, we developed a model of liver carcinogenesis using a choline-deficient L-amino acid (CSAA) diet, in which formation of 8-hydroxydeoxyguanosine (8OHdG), generally accepted as one of the most reliable markers of oxy radical-mediated DNA damage possibly essential for carcinogenesis (26—34), correlates with development of putative preneoplastic gamma-glutamyltransferase (GGT) positive focal lesions (35).

In the present experiment, we studied the effects of dietary iron deficiency on the induction of putative preneoplastic lesions, 8OHdG in DNA and lipid peroxidation in the livers of rats by the CDAA diet since iron is known to be one of the most important catalysts for the formation of highly reactive oxy radicals in a variety of situations (36) and reported to induce 8OHdG in rat kidney DNA when administered i.p. as a form of ferric nitrilotriacetate (34).

Materials and methods

Animals and diets

A total of 200 male Fischer 344 rats were obtained at 3 weeks of age from Japan SLC Incorporated, Hamamatsu, Shizuoka, Japan and used after being acclimatized for 1 week with free access to Oriental MF Diet (Oriental Yeast Company, Ltd, Itabashi, Tokyo, Japan). The animals were housed in iron-free plastic cages in an air-conditioned room maintained at 25°C with a 12-h dark/light cycle and allowed access to food and tap water ad libitum throughout the experiment. After acclimation, eight experimental groups each consisting of 25 rats were designated. The CDAA and a choline-supplemented L-amino acid defined (CSAA) diets, of which compositions were described previously (35), were obtained from Dyets Incorporated, Bethlehem, Pennsylvania (Product numbers 518751 and 518752 respectively). The CDAA diet contained 6.5 mg/kg of choline and 1.75 g/kg of methionine.

The iron content of the CDAA or CSAA diet was 330 p.p.m. while the iron deficient versions of the CDAA and CSAA diets, produced by Dyets (Product numbers 518751 and 518752 respectively), had a value diminished to <5 p.p.m. All diets had a similar caloric content. Diets were stored at 4°C immediately on arrival, and each batch of diets was consumed within 1 month.

Experimental protocol

The experimental period consisted of two parts: 4-week pretreatment and 84-day treatment (periods A and B respectively). Group 1 was given the CSAA and CDAA diets during periods A and B respectively. Group 2 was given the iron-deficient CSAA and iron-deficient CDAA diets during periods A and B respectively. Group 3 was given the CSAA diet during period A and the iron-deficient CDAA diet during period B. Group 4 was given the iron-deficient CSAA diet during period A and the CDAA diet during period B. Groups 5—8 acted as controls for groups 1—4 respectively. In such groups, rats were treated similarly to those in the respective positive experimental groups with the exception that the CSAA and iron-deficient CSAA diets were respectively fed in place of the CDAA and iron-deficient CDAA diets. All rats were weighed weekly during the first 4 weeks of period...
B and biweekly thereafter. Diets were replaced every Monday and Friday when average food intake per rat per day was calculated.

Subgroups of five rats from each group were sacrificed under ether anesthesia at the commencement of period B and 3, 21, 42 and 84 days thereafter. On sacrifice, blood was taken from the bifurcation of the abdominal aorta, and the livers were excised. Sera were fractioned from the blood samples and used for biochemical analyses. The livers were blotted, weighed and utilized for histological, histochemical, immunohistochemical and biochemical analyses.

**Results**

In the livers of rats in group 1, 7.57 GGT and 8.32 GSTP positive lesions were detected per cm² of specimen. Dietary iron deficiency during period A and/or period B in groups 2—4 significantly reduced the numbers of such lesions. As for the size of the enzyme-altered lesions, the mean diameter of lesions in the livers of rats in group 1 was 1.83 mm for GGT and 1.98 mm for GSTP. Whereas size was not altered in group 4, GGT but not GSTP positive lesions in groups 2 and 3 were significantly smaller.

**Inhibition of development of putative preneoplastic liver lesions by dietary iron deficiency**

The results of quantitative analysis of GGT and GSTP positive liver lesions at the end of the experiment are summarized in Table I. In the livers of rats in group 1, 7.57 GGT and 8.32 GSTP positive lesions were detected per cm² of specimen. Dietary iron deficiency during period A and/or period B in groups 2—4 significantly reduced the numbers of such lesions. As for the size of the enzyme-altered lesions, the mean diameter of lesions in the livers of rats in group 1 was 1.83 mm for GGT and 1.98 mm for GSTP. Whereas size was not altered in group 4, GGT but not GSTP positive lesions in groups 2 and 3 were significantly smaller.

**Inhibition of 80HdG formation in DNA and peroxidation of membrane lipids in the liver by dietary iron deficiency**

Results for determinations of 80HdG formation in DNA and lipid peroxidation in the livers of rats at the end of the experiment are shown in Table II. Formation of 80HdG increased in groups 1—4 as compared with the respective values in groups 5—8. The highest level was obtained in group 1 while values in groups 2—4, suffering from dietary iron deficiency in period A and/or period B, were all significantly lower that in group 1. Results for lipid peroxidation displayed the same tendencies as those for 80HdG formation. The data from sequential determinations of 80HdG formation and lipid peroxidation are summarized in Figures 2 and 3 respectively. Both 80HdG formation and lipid peroxidation displayed the same tendencies as those for 80HdG formation. The data from sequential determinations of 80HdG formation and lipid peroxidation are summarized in Figures 2 and 3 respectively. Both 80HdG formation and lipid peroxidation displayed the same tendencies as those for 80HdG formation. The data from sequential determinations of 80HdG formation and lipid peroxidation are summarized in Figures 2 and 3 respectively.
Dietary iron deficiency inhibits rat hepatocarcinogenesis by CDAA diet

peroxidation increased in a time-dependent fashion in groups 1, 2 and 4 from day 3 of period B. In group 3, an increase was observed from day 3 to day 21 of period B with a tendency for plateau levels to be maintained thereafter. Values in groups 1 and 2 were always the highest and the lowest respectively within the dietary choline deficient groups. During period B, values in group 3 were higher on days 3 and 21 and lower on days 42 and 84 than those in group 4.

Histological findings

In addition to GGT and GSTP positive lesions, fatty cirrhosis developed in the livers of all rats in groups 1—4. No histological changes were observed in the livers of rats in groups 5—8.

Serological findings

Data for serum levels of AST and ALT at the end of the experiment are summarized in Table III. Serum activities of AST and

<table>
<thead>
<tr>
<th>Group</th>
<th>Diet</th>
<th>Final body wt (g)</th>
<th>Final liver wt (g/100 g body wt)</th>
<th>GGT positive lesions</th>
<th>GSTP positive lesions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Number of lesions/cm²</td>
<td>Size of lesions (mean diameter; mm)</td>
</tr>
<tr>
<td>1</td>
<td>CSAA</td>
<td>272 ± 9[b]</td>
<td>4.95 ± 0.31[b]</td>
<td>7.57 ± 1.35</td>
<td>1.83 ± 0.10</td>
</tr>
<tr>
<td>2</td>
<td>Iron deficient CSAA</td>
<td>281 ± 7[c]</td>
<td>3.70 ± 0.25[c,d]</td>
<td>3.36 ± 1.01[d]</td>
<td>1.53 ± 0.18[d,g]</td>
</tr>
<tr>
<td>3</td>
<td>CSAA</td>
<td>276 ± 7[d]</td>
<td>3.84 ± 0.15[d,f]</td>
<td>3.30 ± 1.40[g]</td>
<td>1.55 ± 0.15[g,d]</td>
</tr>
<tr>
<td>4</td>
<td>Iron deficient CSAA</td>
<td>282 ± 8[e]</td>
<td>3.77 ± 0.26[e,f]</td>
<td>4.58 ± 1.07[f]</td>
<td>1.85 ± 0.15</td>
</tr>
<tr>
<td>5</td>
<td>CSAA</td>
<td>372 ± 39</td>
<td>3.13 ± 0.20</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>Iron deficient CSAA</td>
<td>360 ± 12</td>
<td>2.98 ± 0.10</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>CSAA</td>
<td>365 ± 22</td>
<td>3.02 ± 0.10</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>Iron deficient CSAA</td>
<td>369 ± 21</td>
<td>3.00 ± 0.12</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*Results shown are the means ± SD of determinations on groups of five rats.

bP < 0.01 versus group 5.
cP < 0.001 versus group 6.
dP < 0.001 versus group 7.
eP < 0.001 versus group 8.
fP < 0.05 versus group 1.
gP < 0.05 versus group 4.

Table II. Inhibitory effect of dietary iron deficiency on induction of oxidative DNA damage and lipid peroxidation in the livers of rats fed the CDAA diet

<table>
<thead>
<tr>
<th>Group</th>
<th>Diet</th>
<th>Oxidative DNA damage (8OHdG/10*dG)</th>
<th>Lipid peroxidation (TBA reactive substances: nmol MDA eq/g wet liver)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>CSAA</td>
<td>17.79 ± 2.22[b]</td>
<td>10.45 ± 0.64[b]</td>
</tr>
<tr>
<td>2</td>
<td>Iron deficient CSAA</td>
<td>6.65 ± 0.52[c,d,b]</td>
<td>4.73 ± 0.18[c,d,b]</td>
</tr>
<tr>
<td>3</td>
<td>CSAA</td>
<td>8.92 ± 0.91[d,f,a,i]</td>
<td>5.55 ± 0.23[d,f,a,i]</td>
</tr>
<tr>
<td>4</td>
<td>Iron deficient CSAA</td>
<td>12.19 ± 1.36[f,a,i]</td>
<td>8.36 ± 0.23[f,a,i]</td>
</tr>
<tr>
<td>5</td>
<td>CSAA</td>
<td>1.34 ± 0.17</td>
<td>0.45 ± 0.12</td>
</tr>
<tr>
<td>6</td>
<td>Iron deficient CSAA</td>
<td>1.42 ± 0.14</td>
<td>0.43 ± 0.08</td>
</tr>
<tr>
<td>7</td>
<td>CSAA</td>
<td>1.25 ± 0.09</td>
<td>0.43 ± 0.14</td>
</tr>
<tr>
<td>8</td>
<td>Iron deficient CSAA</td>
<td>1.36 ± 0.08</td>
<td>0.44 ± 0.10</td>
</tr>
</tbody>
</table>

*Results shown are the means ± SD of determinations on groups of five rats.

bP < 0.01 versus group 5.
cP < 0.001 versus group 6.
dP < 0.001 versus group 7.
eP < 0.001 versus group 8.
fP < 0.05 versus group 1.
gP < 0.05 versus group 3.
hP < 0.05 versus group 4.
iP < 0.05 versus group 2.
ALT were elevated in groups 1–4 as compared with those in groups 5–8. There were no significant differences among groups 1–4.

Discussion

Previously, we reported a correlation between formation of 8OHdG in DNA and development of enzyme-altered lesions in the livers of rats given the CDA A diet (35). Both changes were more severe in the case of the CDA A diet than with a semipurified choline-deficient diet (35). The CDA A diet has the added advantage for the preparation of an iron-deficient version since it does not contain alcohol extracted peanut meal, soy protein isolate or vitamin free casein. As the iron, which is contained in such components, cannot be excluded, it is effectively impossible for its removal from the semipurified choline-deficient diet. The CDA A diet is a marker of ox radicals induced DNA damage, which was discovered by Floyd et al. (41) and Kasai et al. (26–28). Floyd (31) recently gave a commentary about the role of 8OHdG in carcinogenesis and listed several protocols for the assessment of association between 8OHdG formation in DNA or RNA and tumor induction. The importance of 8OHdG formation in DNA is indicated by the finding that its presence in a DNA template causes α-polymerase to miscode incorporation of nucleotides in the replicated strand (29). It has been reported that 8OHdG within DNA is mutagenic (45). Taken together, our previous (35) and present results suggest that 8OHdG induced in liver DNA by the CDA A diet could largely account for the hepatocarcinogenicity of the diet in rats.

Table III. Serum levels of AST and ALT in rats at the end of the experiment

<table>
<thead>
<tr>
<th>Group</th>
<th>Diet</th>
<th>Period A</th>
<th>Period B</th>
<th>AST (U/l)</th>
<th>ALT (U/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CDA A</td>
<td>CDA A</td>
<td></td>
<td>331 ± 32*</td>
<td>229 ± 37*</td>
</tr>
<tr>
<td>2</td>
<td>Iron deficient CDA A</td>
<td>Iron deficient CDA A</td>
<td>283 ± 85*</td>
<td>219 ± 27*</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>CDA A</td>
<td>Iron deficient CDA A</td>
<td>273 ± 87*</td>
<td>217 ± 22*</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Iron deficient CDA A</td>
<td>Iron deficient CDA A</td>
<td>304 ± 22*</td>
<td>213 ± 23*</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>CDA A</td>
<td>CDA A</td>
<td></td>
<td>53 ± 14</td>
<td>24 ± 3</td>
</tr>
<tr>
<td>6</td>
<td>Iron deficient CDA A</td>
<td>Iron deficient CDA A</td>
<td>56 ± 10</td>
<td>30 ± 6</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>CDA A</td>
<td>Iron deficient CDA A</td>
<td>46 ± 14</td>
<td>24 ± 8</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Iron deficient CDA A</td>
<td>Iron deficient CDA A</td>
<td>49 ± 16</td>
<td>26 ± 6</td>
<td></td>
</tr>
</tbody>
</table>

*Results shown are the means ± SDs of determinations on groups of five rats. 

References

1. H. Yoshiji et al. (2019). Inhibition by dietary iron deficiency of the CDA A diet-induced hepatic oxidative DNA damage as a function of time. Results shown are the means plus the standard deviations of determinations on groups of five rats. Group 1 (○) received the CSAA and CDA A diets in periods A and B respectively. Group 2 (△) received the iron-deficient CSAA and iron-deficient CDA A diets in periods A and B respectively. Group 3 (□) received the CDA A diet in period A and the iron deficient CDA A diet in period B. Group 4 (△) received the iron-deficient CSAA diet in period A and the CDA A diet in period B. Groups 5 (●), 6 (△), 7 (□) and 8 (○) acted as controls for groups 1, 2, 3 and 4 respectively. In such groups, rats were treated similarly to those in the respective positive experimental groups with the exception that the CSAA and iron-deficient CSAA diets were respectively fed in place of the CDA A and iron-deficient CDA A diets.

2. H. Yoshiji et al. (2019). Inhibition by dietary iron deficiency of the CDA A diet-induced hepatic lipid peroxidation as a function of time. Results shown are the means plus the standard deviations of determinations on groups of five rats. Group 1 (○) received the CSAA and CDA A diets in periods A and B respectively. Group 2 (△) received the iron-deficient CSAA and iron-deficient CDA A diets in periods A and B respectively. Group 3 (□) received the CDA A diet in period A and the iron-deficient CDA A diet in period B. Group 4 (△) received the iron-deficient CSAA diet in period A and the CDA A diet in period B. Groups 5 (●), 6 (△), 7 (□) and 8 (○) acted as controls for groups 1, 2, 3 and 4 respectively. In such groups, rats were treated similarly to those in the respective positive experimental groups with the exception that the CSAA and iron-deficient CSAA diets were respectively fed in place of the CDA A and iron-deficient CDA A diets.
In the present investigation of the role of iron in the CDAA diet-associated induction of liver preneoplastic lesions, the results strongly suggest mediation by oxy radicals. Furthermore, sequential determination of 8OHaG formation indicated that unrepairable oxidative DNA damage persists and, indeed, accumulates. While it is possible that this might partly be the result of detecting DNA damage in denatured DNAs of necrotic hepatocytes, in the present study, dietary iron deficiency inhibited 8OHaG formation without any marked effect on hepatocellular injury at least when the latter was assessed at the end of the experiment. It is thus suggested that the 8OHaG detected in our previous investigation (35) and in an independent report by Hinrichsen et al. (46), using rats subjected to a two-thirds partial hepatectomy followed by feeding of a semipurified choline-deficient diet for 24 weeks, was similarly not simply a reflection of DNA damage in lethally injured cells. However, further studies of the possible relationship between necrosis and 8OHaG formation are required.

Banni et al. (47,48) claimed that a semipurified choline-deficient diet did not induce hepatocellular membrane lipid peroxidation and that trans fatty acid and fatty acids with conjugated dienes, present in a partially hydrogenated fat, were absorbed and assimilated in hepatic lipids in rats. They also showed that no conjugated dienes were detected if the diet contained no partially hydrogenated fat but only corn oil. Thus, it should be noted that, at the present time, the potential occurrence of hepatic lipid peroxidation in rats fed a choline (or methyl donors) deficient diet has become doubtful. Nevertheless, the present results demonstrated that the TBA reacting substances accumulated in a time-dependent manner in the livers of rats fed the CDAA diet while no such an accumulation was seen in those of rats fed the CSAA diet despite the presence of a partially hydrogenated fat (Primex) at a concentration of 10% in both of the CDAA and CSAA diets, that the CDAA diet-induced accumulation of the TBA reacting substances was inhibited by associated dietary iron deficiency and, finally, that the TBA reacting substances obtained from the samples revealed exactly identical fluorescence spectra to those obtained from the MDA standards. Those demonstrations, therefore, strongly suggest that the feeding of the CDAA diet induces hepatocellular membrane lipid peroxidation in rats. Further studies are necessary to explain the discrepancy between our results and those stated above.

Iron, an essential nutrient for life present in various metal-containing proteins such as haemoglobin, acts as a catalyst in a variety of chemical reactions, becoming oxidized or reduced. In living creatures, including humans, a deficiency in iron is well known to cause anaemia and growth retardation while a surplus in iron results in haemochromatosis. Moreover, iron is reported to take part in the processes underlying carcinogenicity. Human patients suffering from hereditary haemochromatosis have a high risk of hepatocellular carcinoma development (49). Experimentally, growth of various malignant tumour cells requires iron (50,51), and iron overload enhances experimental colon (52) or mammary carcinogenesis (53). In an earlier report, we described an inhibitory effect of dietary iron deficiency upon development of putative preeenzyme-altered hepatocyte foci after initiation with diethylnitrosamine and promotion by phenobarbital in rats (54). It is conceivable that a considerable number of pathological phenomena, if not all, induced by an alteration of iron level in living creatures may be explained in connection with its role in oxidative stress reactions. In fact, a causal participation of oxy radicals has been proposed in the mechanisms underlying the renal carcinogenicity and nephrotoxicity of ferric nitrolotriacetate (34,55) and the hepatotoxicity associated with chronic iron overload (49). In this context, the present inhibitory effect of dietary iron deficiency on 8OHaG formation in DNA, induction of lipid peroxidation and development of GGT and GSTP positive lesions in the livers of rats fed the CDAA diet strongly suggests a causal role for an insufficiency of catalytic iron in the generation of activated oxygen species.

In group 4 of the present experiment, in which dietary iron was diminished only during period A and not during period B, the numbers of GGT positive lesions, 8OHaG formation and lipid peroxidation but not the sizes of the lesions were reduced. Thus, according to Pitot et al. (56), the division of activities into initiating and promoting compartments and our data suggest that the dietary iron deficiency for 4 weeks prior to the CDAA diet exposure modified only the initiation phase. Our previous report indicated that a significant hepatic iron deficiency is gradually achieved after the start of dietary iron deficiency similar to that used in the present experiment and persists for at least 1 week after return to normal iron intake (54). Therefore, the duration of effective exposure to dietary iron deficiency would have been shortened in group 4, explaining the reduction only in the numbers of lesions. In addition, since formation of 8OHaG proved time dependent in a linear way, the results for group 4 would also be expected.

Despite the presence of a number of reports indicating an initiating ability of dietary choline (or methyl donors) deficiency (3,5,23,35,57), it has been argued that it does not initiate rat hepatocytes per se but only promotes pre-existing spontaneously (or endogenously) initiated hepatocytes (9,58). Our data would support, however, the initiating ability of the CDAA diet, dependent on the presence of sufficient iron.

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