Review

A New Hypothesis for Uterine Carcinogenesis: A Pathway Driven by Modulation of Estrogen Metabolism through Cytochrome P450 Induction in the Rat Liver

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Abstract: Estrogen dependence is generally accepted as a cue for mammary and uterine carcinogenesis, but recently estrogen metabolite or catechol-estrogen driven pathways have also come under consideration. Endometrial adenocarcinoma is a leading cause of cancer death in women. Although the cancer is rare in most strains of rodents, it develops spontaneously and can be readily induced in the Donryu rat strain, with many morphological, endocrinological and molecular similarities to the human case. The goal of this review is to weigh the hypothesis for a new pathway for endometrial carcinogenesis driven by modulation of estrogen metabolism through cytochrome P450 enzyme induction using data from the Donryu rat model. To test our hypothesis, indole-3-carbinol (I3C), an active ingredient of cruciferous vegetable, was selected, since it induces cytochrome P450 enzymes in the liver which impact on hydroxylation of estrogens. In uterotrophic assays using ovariectomized rats, neither 500 ppm nor 2000 ppm of I3C in the diet caused any estrogenic or anti-estrogenic activity. However, in our 2-stage rat uterine cancer model, dietary I3C and subcutaneous injection of 4-hydroxyestradiol (4HE), one of hydroxylation metabolites of 17β-estradiol (E2), elevated both incidences of uterine adenocarcinomas and multiplicities of uterine proliferative lesions. I3C treatment increased mRNAs for 1A1, 1A2 and 1B1 in the liver, reflecting the distribution of corresponding enzymes immunohistochemically demonstrated. In the uterus, only CYP 1A1 mRNA was increased by the treatment, without reflecting protein expression. In the liver, I3C consistently elevated estradiol 2 and 4 hydroxylation. These results indicate that modulation of estrogen metabolism, particularly to increase 4HE through induction of CYP 1 in the liver, plays a crucial role in promoting effects of dietary I3C on endometrial adenocarcinoma development, providing support for our hypothesis of a new pathway for endometrial carcinogenesis in the rat. (J Toxicol Pathol 2006; 19: 57–67)

Key words: uterine carcinogenesis, estrogen, metabolism, CYPs, liver, rat

Introduction

The main target of toxicology research is to detect adverse effects of chemicals on living organisms, while generating an understanding of underlying mechanisms to provide a rational basis for: 1) interpreting descriptive toxicity data; 2) estimating the probability that a chemical will cause harmful effects; 3) establishing procedures to prevent or antagonize toxic effects, 4) designing drugs and industrial chemicals that are less hazardous; and 5) developing pesticides that are more selectively toxic for their target organisms.

The liver is the primary target organ for most chemicals since its task is to maintain the body’s metabolic balance. Bioactivation and detoxification, that comprise the major outcome of chemical metabolism, are usually carried out in hepatocytes having high constitutive activities of many phase I and II enzymes, with frequent enhancement of these activities by repeated chemical exposure, as morphologically manifested by hepatocellular hypertrophy. In general, the ratio between phase I and phase II reactions governs whether a compound causes liver cell injury or is safely detoxified.

Recent work has provided evidence that certain metabolic enzyme inducers in the liver exert indirect effects on other organs. For example, phenobarbital (PB), which is a prototype hepatic microsomal inducer that targets a spectrum of cytochrome P-450 isoenzymes (CYPs), also increases activity of uridine diphosphate
glucuronosyltransferase (UDP-GT), the rate-limiting enzyme in T4 metabolism in the rat\textsuperscript{1,2}, and thus leads to a decrease in blood T4\textsuperscript{1,2}. Consequently, activation of the hypothalamo-pituitary-thyroid control system is activated with resultant elevation in the output of thyroid stimulating hormone (TSH). In 2-year or lifetime chronic toxicity/carcinogenicity studies, such long-term stimulation by PB and compensatory elevation of serum TSH levels finally results in thyroid follicular cell tumors\textsuperscript{3,4}. Additionally, recent studies have proved that the decrease in serum thyroxine level by PB in rats is not necessarily dependent on increase in hepatic UDP-GT\textsuperscript{5}.

Induction of CYPs by non-genotoxic carcinogens is not thought to directly cause tumors, but many of the inducible forms are common to those responsible for metabolism of estrogen or other steroids, such as hydroxylation\textsuperscript{6,7}. A number of chemicals such as environmental pollutants and dietary supplements\textsuperscript{8–15} are inducers of such CYPs, especially of the CYP 1 or 3 family. Recently much attention has been paid to 4-hydroxyestradiol (4HE), a hydroxylation metabolite of 17β-estradiol (E2) that have been reported to show stronger carcinogenic potential than E2, its parent compound, with reference to production of DNA damage in humans and animals\textsuperscript{16–19}. This may be of particular importance for endometrial adenocarcinomas in the uterine corpus\textsuperscript{20}, which are generally increasing in women residing in developed countries and are now a leading cause of cancer death. While naturally occurring endometrial adenocarcinomas are generally very rare in rats\textsuperscript{21}, Maekawa et al. found a high incidence of spontaneous lesions in aged Donryu rats with close similarities to human tumors\textsuperscript{22,23}, and have subsequently established a rat model of uterine carcinogenesis\textsuperscript{24}. Elevated binding of E2 or other estrogenic compounds to estrogen receptor (ER)\alpha is also a trigger for uterine carcinogenesis process of rodents\textsuperscript{25–28} (Fig. 1), although precise mechanisms remain to be determined.

The hypothesis assessed in the present review is that chemicals exerting no estrogenic activity themselves but inducing CYPs might modify estrogen-dependent tumor development, and that a new pathway driven by modulation of estrogen metabolism via CYPs inducer might contribute to uterine carcinogenesis in rats. For this purpose I briefly introduce features of endometrial adenocarcinomas in Donryu rats, and present recent supportive data regarding induction of uterine cancers by CYP inducers. Whereas the liver is the main target of estrogen metabolism, in situ modulation of metabolite formation by CYPs in the mammary gland or uterus has also been recently reported\textsuperscript{26}. Therefore a discussion of the possibility of in situ induction of CYPs in the uterus is included.

**Morphological and Endocrinological Features of Endometrial Adenocarcinomas in Donryu Rats**

Endometrial adenocarcinomas morphologically develop through multiple stages in rats, as in the human case\textsuperscript{20,22,23}. In middle-aged Donryu female rats (approximately 10 months of age, and equivalent to the menopausal phase in women) atypical hyperplasias characterized by endometrial epithelial cell hyperplasias with slight cellular atypia begin to develop in the endometrium as localized foci of proliferation, then increasing in number and size, demonstrating cellular atypia,
with aging. In the next step, well-differentiated endometrial adenocarcinomas with morphological similarities to severe atypical hyperplasias but featuring invasion into the muscle layer or spread to the serosa in the uteri and/or the abdominal cavity, can be found. The incidence of such adenocarcinomas finally rises to 30–50% at 24 months of age. Furthermore, some adenocarcinomas demonstrate progression to moderately or poorly differentiated forms, increasing their malignancy. Based on the sequential development, the atypical hyperplasias are recognized as precursors of endometrial adenocarcinomas (Fig. 2).

A characteristic feature strongly linked to uterine carcinogenesis in this strain of rats is ovarian hormonal imbalance leading to elevation of serum E2 levels relative to progesterone (P), in line with uterine proliferating lesion development. This imbalance leads to anovulation, morphologically detectable as atrophic ovaries with polycystic atretic follicles and loss of corpora lutea, eventually resulting in persistent estrus (PE) confirmable by vaginal cytology. PE first appears from 4 months of age in Donryu rats and affects most animals at 11 months, when other rat strains are still capable of reproduction. It should be noted that polycystic ovary is a high risk factor for uterine cancer in women.

Establishment of a Uterine Endometrial Adenocarcinoma Model Using Donryu Rats

Based on the morphological and endocrinological similarities of uterine cancers in Donryu rats to those in women, a 2-stage uterine carcinogenesis model was established by Maekawa’s group to detect promoting or preventive effects of test-chemicals. Briefly, in this model female Donryu rats at 10 or 11 weeks of age are initiated with N-ethyl-N’-nitro-N-nitrosoguanidine (ENNG) at the concentration of 20 mg/kg dissolved in polyethylene glycol and introduced into a unilateral uterine horn via vagina using a stainless steel catheter for initiation. Then the rats are exposed to test materials for 12 months and at 15 months of age are sacrificed to determine incidences or
multiplicities of uterine neoplastic lesions for comparison with controls (ENNG initiation only). The intrauterine treatment with a single dose of ENNG yields higher and earlier development of uterine neoplastic lesions than is the case with spontaneous development and their morphologic features are extremely similarities between spontaneous and induced cases. In addition, the ENNG treatment is specific to the uteri, not affecting ovarian function and other organ tumorigenesis.

**Estrogen Metabolite or Catechol Estrogen Driven Uterine Carcinogenesis**

In the rat liver, E2 is metabolized by estradiol 2- and 4-hydroxylases into the catechol estrogens, 2-hydroxyestradiol (2HE) and 4-hydroxyestradiol (4HE), respectively (Fig. 3). 2-Hydroxylation of estradiol is the dominant pathway for catechol estrogen formation and 2HE can bind to ERα, but with a markedly reduced affinity. This metabolite thus possesses much weaker hormonal potential than the parent hormone, and there is evidence that it is not a carcinogenic agent. In contrast, 4HE, produced only in small amounts in the liver compared to 2HE, is hormonally active and can stimulate uterine growth with strong binding to estrogen receptors when injected into animals. In

![Fig. 3. Major pathway of catechol estrogen metabolism.](image)

**Table 1.** Incidences (%) of Uterine Proliferative Lesions and Their Multiplicities at 15 Months of Age [Modified from ref. 52]

<table>
<thead>
<tr>
<th>None</th>
<th>Atypical hyperplasia</th>
<th>Adenocarcinoma</th>
<th>Multiplicity(a)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Slight</td>
<td>Moderate</td>
<td>Severe</td>
</tr>
<tr>
<td>Control</td>
<td>16.7</td>
<td>8.3</td>
<td>20.8</td>
</tr>
<tr>
<td>I3C 500 ppm</td>
<td>3.3</td>
<td>6.8</td>
<td>10.0</td>
</tr>
<tr>
<td>Control</td>
<td>11.1</td>
<td>11.1</td>
<td>38.9</td>
</tr>
<tr>
<td>I3C 2000 ppm</td>
<td>5.6</td>
<td>11.1</td>
<td>27.8</td>
</tr>
<tr>
<td>E2 1 µg/kg</td>
<td>0</td>
<td>18.8</td>
<td>12.4</td>
</tr>
<tr>
<td>4HE 5 µg/kg</td>
<td>0</td>
<td>0</td>
<td>31.2</td>
</tr>
</tbody>
</table>

(a) Multiplicities are average numbers of uterine proliferative lesions per rat, mean ± SD.

*, **, Significantly different from the relevant control group (p<0.05 and 0.01, respectively. I3C, indole-3-carbinol.

![Fig. 4. Levels of expression of cytochrome P450s 1A1, 1A2 and 1B1 mRNA relative to GAPDH mRNA in the liver (calculated as %) in the rat at 15 months of age. Control, control group given basal diet only; I3C-2000, indole-3-carbinol at 2000 ppm in basal diet. [Modified from ref. 52](image)
addition, this catechol estrogen has been reported to be a stronger carcinogen than the parent E2 due to production of DNA damage\(^6,^{20}\), and causes tumor development in the kidney in hamsters\(^42\), and possibly uterine and mammary gland neoplasia in human beings\(^18,^{19}\).

Recently, a number of dietary supplements extracted from vegetables have been produced, some of which are known to induce CYPs related to estrogen metabolism in the liver and estrogen dependent organs\(^18,^{40,43}\). Since most of these products exert no direct estrogenic activity in target organs, they can only indirectly impact on estrogen dependent organ carcinogenesis through induction of CYPs and consequent modulation of estrogen metabolism. Indole-3-carbinol (I3C), an active ingredient of cruciferous vegetable, is reported to induce the CYP1 family enzymes in the liver\(^44,^{45}\), to thereby influence hydroxylation of estrogens and to suppress\(^39,^{46-49}\) or promote\(^30,^{51}\) carcinogenesis depending on the animal model. Regarding preventive effects, I3C acts as an anti-estrogen and can induce apoptosis, but precise mechanisms remain to be determined.

To determine estrogenic or anti-estrogenic activity of I3C in rat uteri, female Donryu rats were ovariectomized (OVX) at 9 weeks of age, and starting 2 weeks thereafter were assigned to nine groups receiving: only ovariectomy (controls); daily administration of 500 or 2000 ppm I3C in basal diet (I3C 500 or I3C 2000); 1 µg/kg E2 plus I3C 500 or I3C 2000; daily subcutaneous treatment with E2 at a dose of 1 µg/kg, and E2 metabolites such as 4HE at 5 µg/kg; 2HE at 5 µg/kg and 16α estradiol at 1 µg/kg\(^52\). After 2 weeks treatment, neither dose of I3C was found to have affected uterine weight or the height of the luminal epithelium in ovariectomized rats, with or without E2, and the estrogenic activity of 5 µg/kg 4HE was comparable to 1 µg/kg E2 treatment, while 2HE treatment had no activity. In addition, long-term treatment with dietary I3C at 500 or 2000 ppm did not disturb estrous cyclicity in Donryu rats, clearly demonstrating a lack of any estrogenic or anti-estrogenic activity, in line with results of uterotrophic assays in OVX rats\(^52\).

To clarify the effects of I3C on uterine adenocarcinoma development, 500 or 2000 ppm doses were administered to Donryu rats after ENNG initiation. The incidences and/or the multiplicities of lesions were significantly increased compared with those of the control group, as with 4-HE (Table 1)\(^52\). All uterine adenocarcinomas were of well-differentiated type, and morphological or biological malignancy was not influenced by the I3C treatment.

Histologically, hypertrophy of centrilobular hepatocytes was found in I3C treated groups, along with significantly increased CYP1A1, and to a lesser extent CYP1A2 and 1B1 mRNA expression compared to the control group (Figs. 4, 5). Immunohistochemically, CYP 1A1 and 1A2 were clearly demonstrable in the hepatocytes of centrilobular areas in the liver in both I3C-treated groups (Fig. 6), while mRNA and/or immunohistochemical findings for other cytochrome P450s such as 2B1, 3A1/2 or

**Fig. 5.** mRNA Expression of cytochrome P450s 1A1, 1A2 1B1, 3A1 and 3A2 and GAPDH (Left figure), and levels relative to GAPDH mRNA in the liver (calculated as % values) in ovariectomized rats (Right one). Control, control group given basal diet only; 4HE, daily subcutaneous injection of 5 µg/kg 4-hydroxyestradiol for 2 weeks; I3C-500 and I3C-2000, dietary indole-3-carbinol at 500 or 2000 ppm for 2 weeks, respectively; and I3C-2000+E2, concurrent treatment with I3C-2000 and daily subcutaneous injections of 1 µg/kg E2 for 2 weeks.
Fig. 6. Immunohistochemical localization of CYP1A1, 1A2, 1B1 and 3A2 in the livers of ovariectomized rats. a–d, Control group. e–h, dietary 2000 ppm I3C treated group. Note that centrilobular hepatocytes are strongly positive to CYP1A1 and 1A2, and slightly positive for 3A2, but negative for 1B1. Positive areas are visualized by diaminobenzidine, and counterstaining is with hematoxylin. ×100.

Fig. 7. Sequential changes in enzyme activity related to estrogen metabolism in the liver. Control, control group given basal diet only; I3C-2000, indole-3-carbinol at 2000 ppm in basal diet; E2, subcutaneous injection of 17β-estradiol at 1 μg/kg; 4HE, subcutaneous injection of 4-hydroxyestradiol at 5 μg/kg.
Fig. 8. Sequential changes in enzyme activity related to estrogen metabolism in the livers of ovariectomized rats. Control, control group given basal diet only; E2, subcutaneous injection of 1 µg/kg 17β-estradiol for 2 weeks; 2HE, subcutaneous injection of 5 µg/kg 2-hydroxyestradiol for 2 weeks; 4HE, subcutaneous injection of 5 µg/kg 4-hydroxyestradiol for 2 weeks; I3C-2000, indole-3-carbinol at 2000 ppm in basal diet for 2 weeks and I3C-2000+E2, concurrent treatment with I3C2000 ppm and E2.

Fig. 9. mRNA Expression of cytochrome P450s 1A1, 1A2, 1B1, 3A1 and 3A2 and GAPDH in the uteri of ovariectomized rats. Control, control group given basal diet only; E2, Daily subcutaneous injection of 1 µg/kg 17β-estradiol for 2 weeks 4HE, daily subcutaneous injection of 5 µg/kg 4-hydroxyestradiol for 2 weeks; 2HE, daily subcutaneous injection of 5 µg/kg 2-hydroxyestradiol for 2 weeks; I3C-500 and I3C-2000, dietary indole-3-carbinol at 500 or 2000 ppm for 2 weeks, respectively; and I3C-2000+E2, concurrent treatment with I3C-2000 ppm and E2.
2C6 in the livers were comparable to the control case\(^5\). Measurement also demonstrated increase for estadiol 2- and 4- but not 16α-hydroxylase activities related to estrogen metabolism (Figs. 7 and 8)\(^5\). In both of studies, 4-hydroxylation was particularly significantly increased.

**In Situ Expression of CYPs in the Uterus**

Recently, extrahepatic CYP induction related to estrogen metabolism has been a focus of attention regarding the relationship to tumorigenesis in hamster kidney and human uterine tissue or myoma development\(^18,19,42\). In the hamster kidney, estrogen 2-hydroxylation is catalyzed by members of both CYP 1A and 3A families, the latter known to be related with steroid metabolism. CYP 1B1, which catalyzes E2 to 4-hydroxylation of estradiol, is a candidate key enzyme for development of human myomas. We have investigated CYP 1A1/2, 1B1, and 3A1/2-mRNA expression in the uterus of ovariectomized Donryu rats treated with dietary I3C at 2000 ppm for 2 weeks\(^5\), but no alteration in CYP 1A2, 1B1, 3A1 or 3A2 was observed (Figs. 9, 10). CYP 1A1 mRNA, but not its protein, was slightly up-regulated (Fig. 11). Additional studies of estradiol 4-hydroxylase activity or DNA adduct formation in the uterus need to be performed for confirmation, but the results do strongly suggest that I3C treatment does not affect in situ expression of CYPs in the rat uterus.

**Exploration of a New Pathway for Human Uterine Carcinogenesis**

Induction of CYPs is not considered a direct trigger for promoting effects of non-genotoxic carcinogens. While we have no direct proof that the new pathway described in this review indeed contributes to human uterine carcinogenesis, CYP 1A1, 1A2 and 1B1 show high homology in their functions and control systems among mammals. In addition, a number of studies have indicated that CYP1B1 is a key enzyme for 4HE production through metabolism of E2 in both humans\(^5\) and animals\(^8,15\). We have yet to determine which CYP enzyme is the most important for modulation of estrogen metabolism but further investigation of this area would appear to be clearly warranted.

In conclusion, our finding that I3C treatment promotes uterine endometrial adenocarcinoma development in the Donryu rat provides support for a new hypothesis of uterine carcinogenesis driven by modulation of estrogen metabolism in the liver due to CYP 1 family inducers which exert no estrogenic activity by themselves. The promoting effects are likely due to acceleration of 4-hydroxylation of E2, resulting in 4HE binding to ERs to afford a driving force for carcinogenic responses (Fig. 12).

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