Sex Hormones and Tumor Promotion in Liver (43305)

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Abstract. **Epidemiological and experimental data strongly support a causal relationship between exposure to excessive levels of estrogens and the development of cancer in various tissues. In this paper, we have presented background information that shows a correlation between the prolonged use of oral contraceptives and the development of liver cancer. The clinical data supported the hypothesis that the estrogenic components of oral contraceptives were promoters of hepatocarcinogenesis, and the experimental evidence in support of this hypothesis and bearing on the mechanisms involved are also reviewed. The effects of estrogens on liver neoplasia and growth are: (i) synthetic steroidal estrogens are potent promoters of hepatocarcinogenesis in female rats; (ii) these estrogens stimulate liver growth at doses that are not hepatotoxic; (iii) the mechanisms by which the estrogens stimulate liver growth are indirect and include the enhancement of a serum/plasma growth factor, co-mitogenic effects which result in enhanced responsiveness of cultured hepatocytes to epidermal growth factor and decreased sensitivity of hepatocytes to growth inhibition by transforming growth factor-β; (iv) the co-mitogenic effects of synthetic estrogens extend to endogenous estrogens and natural product estrogens; and (v) the co-mitogenic effects of estrogens for epidermal growth factor are associated with increased epidermal growth factor receptor protein levels caused by an increase in the half-life of the receptor protein. The synthetic estrogens also have weak "complete" carcinogenic activity in rat liver and strong complete carcinogenic activity in Syrian hamster kidney and Armenian hamster liver. Evidence from the literature is presented in support of a hypothesis that this process may involve indirect genotoxicity mediated through redox cycling and the formation of hydroxylated DNA bases. This process, together with the potent promoting activity of these estrogenic chemicals, may account for their complete carcinogenicity.**

[P.S.E.B.M. 1991, Vol 198]

Henderson *et al.* (1) recently reviewed the epidemiological data that support a causal relationship between excessive estrogen exposure from endogenous and exogenous sources and the development of breast and endometrial cancer in humans. With regard to mechanism, they hypothesized that the proliferative effects of estrogen on these target tissues created an opportunity for an increased frequency of replication-associated accidents to occur that would lead to neoplasia. The role of cell proliferation in the carcinogenic effects of nongenotoxic agents such as hormones and various xenobiotics represents an area of investigation that is receiving increased attention (2). This will also become evident in the results presented below, as well as in other papers presented in this symposium.

Liver represents another tissue in which exposure to excessive levels of estrogens has been found to be causally associated with the appearance of neoplasms, both benign and malignant. Initially, evidence suggesting this association was revealed in a report by Baum *et al.* (3), which demonstrated an association between oral contraceptive (OC) use and the appearance of liver adenomas. Subsequently, this association has been well-documented in a number of case-control studies and extended to include the appearance of hepatocellular carcinomas (4). The estrogenic component of OC preparations was considered to represent the active ingredient.

At the time we became interested in these clinical observations, the available experimental evidence indicated that in some instances, cessation of OC use was associated with regression of the adenomas (5). In ad-

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0037-9727/91/1982-0667$3.00/0

dition, studies in which the mutagenicity of the synthetic estrogens had been examined demonstrated a lack of mutagenic activity (6). These observations were consistent with the possibility that the synthetic estrogen components of OC preparations, generally mestranol or ethinyl estradiol, were acting as tumor promoters. Thus, we embarked on a research program aimed at testing the hypothesis that in the rat, these estrogens were promoters of hepatocarcinogenesis. This paper represents a review of our work in this area, together with some new preliminary information pertaining to the mechanism of induction of liver growth by synthetic and endogenous estrogens.

Materials and Methods

Most of the data presented below have been described previously and the original papers should be consulted for experimental details (7–12). All the studies were conducted using intact female rats or hepatocytes derived from them.

Results

Promotion of Hepatocarcinogenesis by Estrogens. Figure 1 illustrates the initiation-promotion protocol used in our studies which was adapted from the protocol described by Pitot and co-workers (see Ref. 13 for references). Female rats were treated by intraperitoneal injection with the initiating agent, diethylnitrosamine, 24 hr after a surgical 2/3 hepatectomy, which provided a proliferative stimulus. Treatment with estradiol, ethinyl estradiol (EE2), and mestranol was begun on the next day in early studies (7, 8) or after a 1-week recovery period, as shown in the Figure 1 (9). Appropriate non-diethylnitrosamine-initiated and non-promoter-treated groups were also included. At 4 months, animals were sacrificed and their livers were analyzed for the presence of altered hepatocellular foci (AHF), as indicated by detection of γ-glutamyl transpeptidase-positive foci, while at later time points, 9 and 12 months, γ-glutamyl transpeptidase-positive AHF, as well as more advanced nodules and hepatocellular carcinomas, were also detected (8).

Figure 2 shows the number of AHF-liver detected in an experiment designed to evaluate whether estradiol, mestranol, and ethinyl estradiol were promoters of hepatocarcinogenesis (8). The estrogens were mixed in the AIN 76A diet at 0.5 ppm; one group was also fed mestranol at 0.1 ppm. In the non-diethylnitrosamine-initiated (saline-treated) animals fed control diet, no AHF were detected; however, both EE2 and mestranol caused a significant increase in AHF number, while the increased number detected with estradiol treatment was not significantly greater than control. These results suggested that the synthetic estrogens alone were either weak complete carcinogens or were promoting the appearance of spontaneously initiated

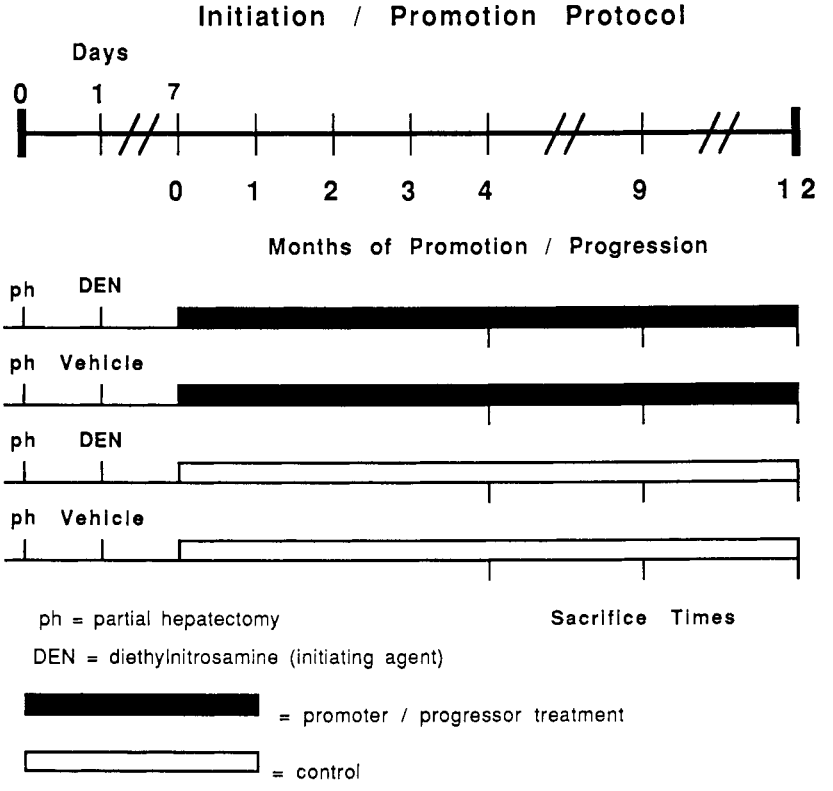

Figure 1. This scheme shows the protocol used in studies designed to determine the promoting activity of the synthetic estrogens for hepatocarcinogenesis (7–9).

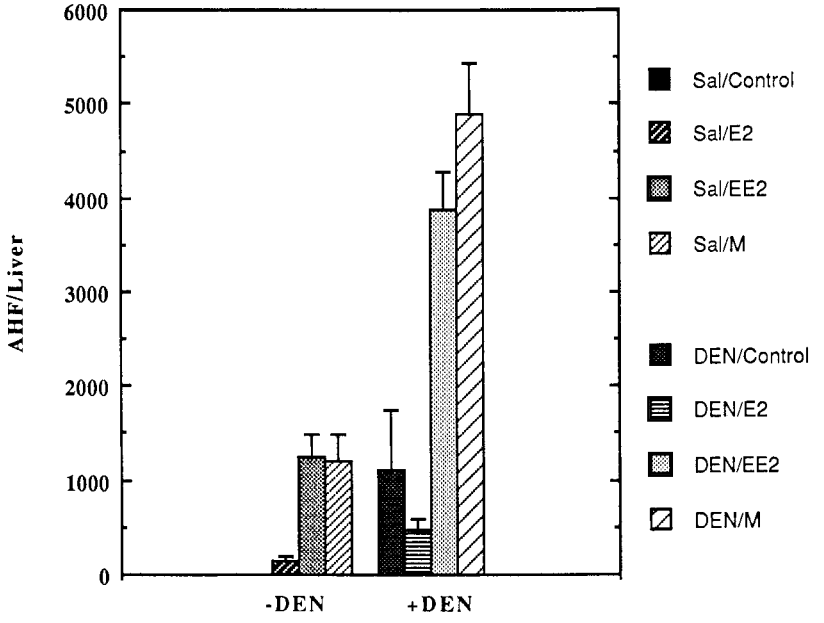

Figure 2. The effects of estradiol (E2), EE2, and mestranol (M) on the appearance of AHF in the livers of female rats. These data were redrawn from Table I in Yager *et al.* (8) and represent the number of γ-glutamyl transpeptidase positive foci calculated to be present per liver after 9 months. Diethylnitrosamine-initiated (+DEN) and -noninitiated (−DEN) rats were fed diet containing the estrogens at 0.5 ppm, as described (8).

hepatocytes (14). Figure 2 also shows the number of AHF in diethylnitrosamine-initiated rats. Both EE2 and mestranol significantly enhanced the appearance of foci, demonstrating their promoting activities. For mestranol, the number of AHF showed a dose response, and the number of rats with liver nodules and hepatocellular carcinomas was significantly increased at both 9 and 12 months (8). In contrast, estradiol, at a comparable dose, did not enhance the appearance of foci.

Recently, Pitot *et al.* (see references in Ref. 13) described a method for calculation of a promotion index that is useful for comparing the relative potencies of chemicals to promote hepatocarcinogenesis in the rat. Figure 3 shows the promotion indices for several chemicals plotted as the log values of data presented by Dragan *et al.* (15). This figure dramatically illustrates that the promotion index values can range over 7 logs, with the peroxisome proliferating agent WY-14,643 having the lowest and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin having the highest activities. While not nearly as potent as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, the synthetic estrogens are clearly very strong promoters.

Mechanisms of Promotion by Estrogens. Table I shows several possible mechanisms of tumor promotion based on the ability of promoters to alter cell proliferation. In this scheme, promoters are categorized as chemicals that either stimulate or inhibit cell proliferation, and selected examples are given. Experiments were conducted to determine the effects of EE2 and mestranol on liver DNA synthesis *in vivo*. It was found that these synthetic estrogens exhibited a dose-dependent ability to stimulate DNA synthesis (9). Following

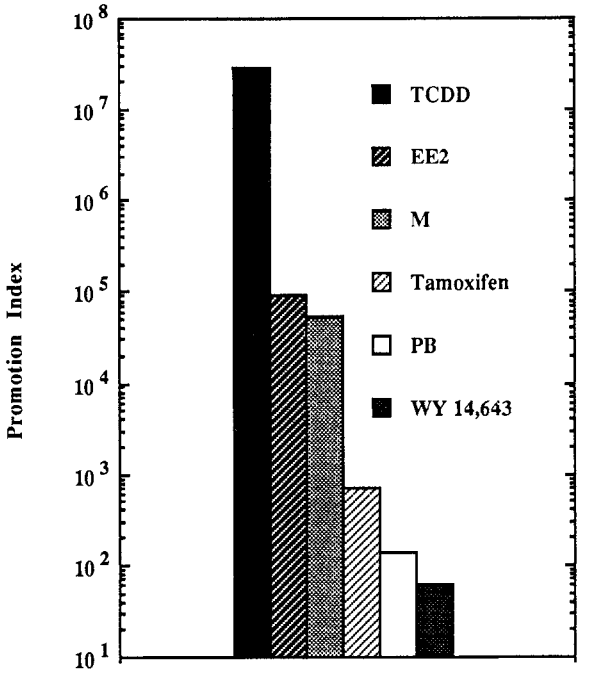

Figure 3. The promotion index of EE2 and mestranol (M) compared with other liver tumor promoters. This figure represents data presented by Dragan *et al.* (15) in Table IV and redrawn on a log scale. Abbreviations used in figure: TCDD,2,3,7,8-tetrachlorodibenzo-*p*-dioxin; PB, phenobarbital; WY 14,643, the peroxisome proliferator [4-chloro-6(2,3-xylidino)-2-pyrimidinylthio]acetic acid.

treatment with the estrogens, DNA synthesis, as detected using a 2-hr pulse of [^{3}H]thymidine, was increased approximately 4-fold within 24 hr, remained elevated through 7 days, and was back to control levels

Table I. Possible Mechanisms of Tumor Promotion

1. Chronic growth stimulation
   A. Hepatotoxicity → restorative hyperplasia
      Choline/methionine deficiency (16,17)
   B. Additive hyperplasia
      Peroxisome proliferators (18)
2. Growth inhibition accompanied by growth stimulation
   A. Resistant hepatocyte model (19)
   B. Orotic acid model (20)

at 14 and 21 days. Thus, the synthetic estrogens are clearly growth stimulatory. Next, we determined whether liver growth is due to hepatotoxicity or additive growth. This was done using the prelabel turnover technique, which is a sensitive method to detect cumulative hepatotoxicity. Rat liver DNA was prelabeled with [^{3}H]thymidine during regenerative hyperplasia following surgical partial hepatectomy (9). After a subsequent 2-week recovery period, the animals were treated with EE2 or mestranol for 6 weeks, at which point the amount of [^{3}H]thymidine remaining per liver was determined. No prelabel loss from liver DNA was detected, indicating the absence of hepatotoxicity of the synthetic estrogens at doses used in the promotion protocol. Thus, the increased liver DNA synthesis detected in EE2- and mestranol-treated rats represents additive hyperplasia (Table I).

For hyperplasia to be mechanistically important in tumor promotion, it would seem necessary that it be persistent. However, as discussed, increased DNA synthesis in response to EE2 was no longer detectable by 14 days. Similar results were observed by Marsman *et al.* (18) for the peroxisome proliferator WY-14,643 when, as in our studies, DNA synthesis was determined using 2-hr pulses with [^{3}H]thymidine. However, when [^{3}H]thymidine was administered by an osmotic minipump over a 7-day period, Marsman *et al.* (18) found that liver DNA synthesis was persistently increased 5- to 10-fold over that of controls in the WY-14,643-treated rats. While it now becomes very important to explain the presence of a chronic increase in hyperplasia induced by WY-14,643 in the absence of continued liver growth (18), the results of Marsman *et al.* clearly point out the importance of the sensitivity of the assay used to detect DNA synthesis *in vivo*. The persistence of liver cell proliferation in response to EE2 is currently being reexamined by using osmotic minipumps to deliver bromodeoxyuridine for subsequent immunohistochemical detection of labeled hepatocytes (21). However, it is also possible that, with chronic exposure, the synthetic estrogens might become mitoinhibitory for "normal" hepatocytes and that AHF represent the clonal outgrowths of initiated hepatocytes resistant to this inhibition. Two observations make this a possibility. First, while phenobarbital initially stimulates liver DNA synthesis (9), Eckl *et al.* (22) have shown that upon chronic exposure, it becomes mitoinhibitory and hepatocytes from phenobarbital-treated animals become less epidermal growth factor (EGF) responsive. Second, Epe *et al.* (23) have shown that diethylstilbesterol and EE2 are metabolized to metabolites that bind to microtubular proteins. It is possible that this could result in mitoinhibitory effects. Clearly, additional studies are required to elucidate the effect of chronic estrogen exposure on liver DNA synthesis.

Mechanisms of Liver Growth Stimulation by Estrogens. Table II lists two primary mechanisms, direct and indirect, that result in liver growth. The various peptide growth factors listed are each able to stimulate DNA synthesis directly in cultured hepatocytes (24). Alternatively, liver growth caused by various xenobiotic stimuli may be mediated by indirect effects, such as induction of a growth factor, co-mitogenic activity, or inhibition of the effects of an inhibitory growth factor such as transforming growth factor (TGF)-β.

Previously, we reported preliminary evidence that EE2 treatment enhanced the serum-plasma levels of a factor(s) stimulatory for DNA synthesis in cultured hepatocytes (25). The identity of this factor(s) is not yet known and efforts are just beginning to determine if it is a hepatocyte growth factor (24). Thus, one component of growth stimulation by EE2 appears to consist of the stimulation of the production and release to the plasma of a direct-acting hepatocyte growth factor.

To determine whether estrogens have direct or indirect growth stimulatory activity on hepatocytes, we employed rat hepatocytes in primary culture. In these studies, hepatocytes were isolated by collagenase perfusion and cultured in collagen-coated dishes in phenol red-free, serum-free medium consisting of Ham's F12-Dulbecco's minimum essential medium (1:1) plus insulin-selenium-transferrin, pyruvate, proline, and gentamycin (10). The cells were allowed to attach for 4 hr (zero time) and subsequently treated with various test agents. DNA synthesis was measured in cultures harvested at 48 hr after exposure to [^{3}H]thymidine. Figure 4 shows the effects of EE2, EGF, and both together on DNA synthesis in cultured hepatocytes (10). EE2 alone

Table II. Mechanisms of Liver Growth Stimulation[a]

1. Direct growth stimulation
   EGF, TGF-α, HGF, aFGF, HPTB, HSS (24)
2. Indirect growth stimulation
   A. Induction of a growth factor(s)
   B. Co-mitogens:
      NE, vasopressin, angiotensins II and III, and insulin (24)
   C. Inhibition of negative growth regulation by TGF-β:
      NE, vasopressin, and angiotensins II and III (24)

[a] Abbreviations used in the table: HGF, hepatocyte growth factor; aFGF, acidic fibroblast growth factor; HPTB, hepatopoietin B; HSS, hepatocyte stimulatory substance; NE, norepinephrine.

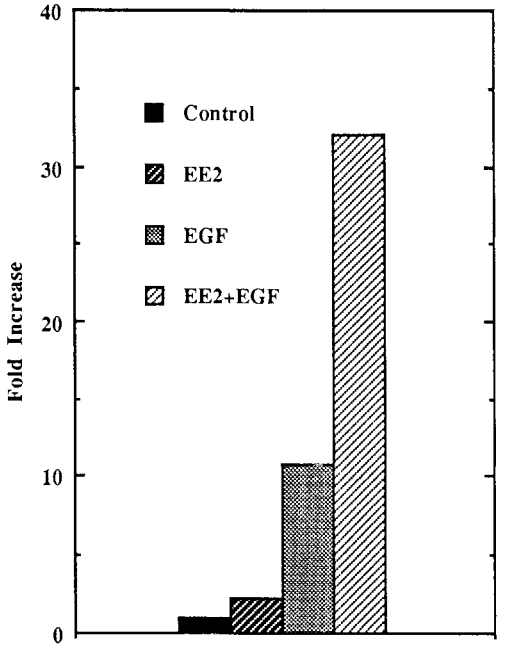

Figure 4. The effects of EE2, EGF, and both together on DNA synthesis in cultured rat hepatocytes. This data was calculated from data presented by Shi and Yager (10) in Table I. The hepatocytes were cultured as described (10) and DNA synthesis was detected by the incorporation of [^{3}H]thymidine into DNA after exposure of the cells to 15 μM EE2 ± 20 ng/ml of EGF for 48 hr.

(15 μM) caused only a 2-fold enhancement in DNA synthesis, whereas the increase was 11-fold for EGF, indicating that EE2 is not a direct-acting hepatocyte growth factor. However, in cultures treated with EE2 + EGF, DNA synthesis was enhanced 32-fold over control levels. This increase was greater than 2 times the additive effects of each alone. Statistical analysis of these data indicated a significant interaction between EE2 and EGF in their effects on hepatocyte DNA synthesis (10). Additional studies demonstrated that pretreatment of the hepatocytes prior to the addition of EGF also enhanced EGF responsiveness and that this effect was associated with an increase in specific ^{125}I-EGF binding (10). This effect was dependent on the concentration of EE2, with maximum enhancement occurring between 10 and 15 μM. Subsequent studies demonstrated that EE2 was rapidly metabolized by the hepatocytes, becoming undetectable by high performance liquid chromatography analysis within 4 hr of addition to the cultures (12). Additional studies also demonstrated that the increase in ^{125}I-EGF binding was associated with an increase in EGF receptor protein and that this increase could be accounted for by an increase in the half-life of the EGF receptor protein (11). In recent preliminary studies, we have shown that the natural estrogen, estradiol, also enhances the EGF responsiveness of cultured hepatocytes. However, higher concentrations of estradiol are required, compared with EE2. This may explain the lack of promoting activity of estradiol (Fig. 2). Together, these results demonstrate that EE2 and estradiol have co-mitogenic effects and, thus, should be added to the list of agents in this category in Table II.

As also indicated in Table II, other co-mitogens have been shown to decrease the DNA synthesis inhibitory activity of TGF-β in cultured hepatocytes. In preliminary studies, we have also determined the effects of estradiol on DNA synthesis inhibition by TGF-β. Hepatocytes were treated with EGF ± estradiol + TGF-β. The results for a single concentration of TGF-β are shown in Table III. These data show that the actual level of EGF-induced DNA synthesis was enhanced by estradiol and that, in the presence of TGF-β, the amount of DNA synthesis occurring in the presence of estradiol was greater than in the absence of estradiol. However, the percentage of inhibition by TGF-β was 60% for EGF alone and 49% for estradiol + EGF. In fact, the percentage of inhibition curves as a function of TGF-β concentration for EGF alone and estradiol + EGF were nearly superimposable (not shown). This suggests that while the amount of DNA synthesis in the presence of TGF-β may be greater if estradiol is present, it is most likely only due to the enhanced EGF responsiveness, rather than to a specific inhibitory effect of estradiol on the TGF-β signal transduction pathway. We also observed similar results with norepinephrine (not shown; 26). Thus, estradiol can also be classed as an inhibitor of negative growth regulation by TGF-β.

Effects of other Synthetic and Endogenous Estrogens and Natural Product Xenobiotics with Estrogenic Activity. In addition to studies on the synthetic steroidal estrogens EE2 and mestranol and the endogenous natural estrogen, estradiol, we have recently conducted preliminary experiments designed to determine the effects of the synthetic estrogens mestranol and diethylstilbesterol (DES), the natural product estrogen α-zearalenol, which is produced by several species of the fungus Fusarium (27), and the endogenous E2 metabolites estrone and estriol on EGF-induced hepatocyte DNA synthesis. Our preliminary results indicate that all of these estrogenic chemicals enhance hepato-

Table III. The Effects of Estradiol on Inhibition of Hepatocyte DNA Synthesis by TGF-β[a]

Treatment	dpm (10^3)/culture[b]
Control	63.5 ± 5.4
Estradiol	139.7 ± 0.9
EGF	546.2 ± 9.2
EGF + TGF-β	217.2 ± 7.8
Estradiol + EGF	987.1 ± 25.3
Estradiol + EGF + TGF-β	504.5 ± 8.8

[a] DNA synthesis was determined in hepatocyte cultures exposed to the EGF (20 ng/ml), TGF-β (0.1 ng/ml), and estradiol (30 μM) for 48 hr. [^{3}H]Thymidine was present from 24 to 48 hr.
[b] Mean ± range of duplicate cultures.

cyte EGF responsiveness (data not shown). At maximally effective doses, their potencies show a rough correlation with their reported estrogenicities, although additional studies are required and under way to confirm this relationship. However, based on these preliminary observations in the cultured hepatocyte system, we hypothesize that at the appropriate doses *in vivo*, these estrogenic compounds would stimulate liver DNA synthesis and also promote hepatocarcinogenesis. Studies are also under way to address this hypothesis.

Discussion

The major findings from the studies conducted in our laboratory and discussed above are summarized in Table IV. The promoting activity of synthetic steroidal estrogens has been clearly shown by our studies and those of others (7–9, 28). However, the appearance of AHF in noninitiated animals treated with EE2 and mestranol, observed in our studies, has not been adequately investigated. As mentioned above, the synthetic steroidal estrogens are not mutagenic. In addition, studies done in our laboratory (29) and by Schuppler *et al.* (30) have shown that the synthetic estrogens are not strong initiators of hepatocarcinogenesis. Furthermore, we also showed that EE2 and mestranol did not cause liver DNA damage *in vivo*, nor did they cause unscheduled DNA synthesis in cultured rat hepatocytes (29). Taken together, these results demonstrated that these agents are not genotoxic in the "classical" sense, and supported the conclusion that the AHF detected in noninitiated rats were due to the promotion of "spontaneously" initiated hepatocytes by these strong promoters (8). However, other explanations for these results are possible. The steroidal estrogens, natural and synthetic, and the nonsteroidal DES have been shown to undergo redox cycling and, in the process, to generate various reactive oxygen species, including hydroxyl radicals (31). The hydroxyl radical can react with DNA to produce hydroxylation products at various sites, including the C-8 position of guanosine (8-OH-dG). Recently, it has been shown that *in vitro*, DES can undergo peroxidative metabolism, resulting in the formation of 8-OH-dG (32). It is possible that, upon chronic exposure to tumor-promoting doses of the synthetic steroidal estrogens, redox cycling occurs and results in the formation of hydroxylated bases. With time, such damage could cause mutations leading to initiation of hepatocarcinogenesis. We currently have a study under way to determine whether chronic treatment with EE2 causes an increase in the basal level (33) of 8-OH-dG detectable in liver.

Table IV. Summary of the Effects of Estrogens on Liver Neoplasia and Growth

1. Synthetic steroidal estrogens are strong promoters of hepatocarcinogenesis.
2. Synthetic steroidal estrogens stimulate liver growth at nonhepatotoxic doses.
3. Estrogen stimulation of liver growth occurs through indirect mechanisms which include:
   A. enhanced levels of a serum-plasma growth factor(s).
   B. co-mitogenic effects as determined with EGF.
   C. inhibition of growth inhibitory effects of TGF-β.
4. The co-mitogenic effects of synthetic estrogens extends to endogenous estrogens and natural product estrogenic chemicals.
5. The co-mitogenic effects of estrogens for EGF are associated with increased EGF receptor levels mediated by increased receptor protein half-life.

In addition to rat liver, the synthetic estrogens, particularly DES, have been shown to induce the appearance of kidney tumors in the Syrian hamster (for references, see Ref. 34). In this model system, DES acts as a "complete" carcinogen, since no prior treatment with an initiating agent is required. However, use of the very sensitive ^{32}P postlabeling technique has not detected the presence of DES-DNA adducts in the kidneys of treated animals (35), although in one recent report a previously undetected proposed DES-DNA adduct was detected shortly after treatment with DES at 200 mg/kg body wt (36). DES has, however, been shown to induce the binding of indigenous compounds to target tissue DNA (35). The formation of such compounds, along with postulated hydroxylated bases, appears to represent indirect genotoxic effects of the estrogens and may play a role in their complete carcinogenic activities in the liver and kidney, but further investigation is required to unequivocally prove a cause and effect relationship.

Interestingly, EE2, while equal to DES in estrogenic potency, has only a weak ability to induce kidney tumors in the Syrian hamster (34). EE2 has been shown to cause liver tumors in the Syrian hamster (37). However, this only occurs when EE2 is administered along with α-napthoflavone, an inhibitor of cytochrome P-450. Finally, DES alone has also been shown to induce hepatocellular carcinomas in Armenian hamsters (38). It is important to point out that in these hamster models for renal and hepatocellular carcinogenesis, the dose of synthetic estrogens used is generally at least 10 times greater than the 2.5- to 5.0-μg/day dose used in the rat to promote hepatocarcinogenesis (7–9).

Our results clearly show that the estrogens stimulate liver growth. However, while this effect occurs through indirect mechanisms which involve the stimulation of an endogenous serum-plasma growth factor along with co-mitogenic effects, further work is necessary to define the endogenous primary growth factors involved, e.g., the identity of the serum-plasma growth factor. In addition, EGF itself is not thought to be important in liver regeneration. It is much less effective in stimulating hepatocyte DNA synthesis than TGF-α (unpublished observations), which does seem to be

involved in liver growth *in vivo* (39). Thus, it becomes important to determine whether the estrogens also enhance the effectiveness of the serum-plasma growth factor they stimulate and of TGF-α. Studies are under way to address these issues. Finally, it is important to determine the nature of the alterations that permit initiated hepatocytes to continue to grow in the presence of chronic synthetic estrogen treatment while normal noninitiated hepatocytes do not.

Recently, Cohen and Ellwein (2) proposed to begin classification of chemical carcinogens based on whether they are DNA reactive (genotoxic) or not (nongenotoxic). The nongenotoxic carcinogens are then classified as to whether or not they interact with a cellular receptor to stimulate cell proliferation. According to this classification scheme, estrogenic carcinogens would be classified as nongenotoxic. The results reviewed above clearly suggest the estrogens are not directly genotoxic and that, for liver, they exert indirect proliferative effects that do not involve toxicity and are most likely mediated through the estrogen receptor. This would classify them as carcinogens which would be effective at low doses, but, in contrast to what is implied by Cohen and Ellwein (2), this would also imply a dose below which no biological effect would occur. In the absence of any direct or indirect genotoxic effects, neoplasms arising upon chronic exposure to this class of carcinogens would only originate from spontaneously transformed cells. However, as discussed above, estrogen metabolites are likely to cause DNA damage mediated through reactive oxygen species or other free radicals, such as organic hydroperoxides generated during estrogen metabolism. The presence of such indirect genotoxicity accompanied by receptor-mediated proliferative effects can thus make these agents potential potent carcinogens, depending upon the extent of their metabolism and the level of estrogen receptors in various tissues. Such differences may account for the differences in their complete carcinogenicity among rat liver, Syrian hamster kidney, and Armenian hamster liver, described above. Perhaps the classification of Cohen and Ellwein (2) should be amended to include indirect genotoxicity. Additional experimentation is also required to determine if other "nongenotoxic" carcinogens, such as the peroxisome proliferators, might also fall into this indirect genotoxic-cell proliferator class.

The research from the authors' laboratory has been supported by National Cancer Institute Grant CA 36701.

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