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Oral Contraceptive Steroids as Promoters or Complete Carcinogens for Liver in Female Sprague-Dawley Rats

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Published reports have described an increased incidence of adenomas and of hepatocellular carcinomas in livers of women with a history of long-term oral contraceptive use. Evidence derived from human and experimental animals suggests that oral contraceptive steroids may be liver tumor promoters.

Experiments were designed to determine the initiating and/or promoting potential of two oral contraceptive steroids, namely mestranol and norethynodrel. The results show that: mestranol and norethynodrel can promote DEN-initiated hepatocarcinogenesis, as indicated by increased numbers of γ-glutamyl transpeptidase-positive, putative preneoplastic lesions and by the appearance of carcinomas. Various synthetic estrogens fail to cause detectable levels of DNA damage in hepatocytes. Mestranol has weak, if any, initiating potential. Neither mestranol nor norethynodrel exhibits antiglucocorticoid activity. Mestranol and norethynodrel inhibit metabolic cooperation in V-79 Chinese hamster cells. Taken together, these results and those reported by others demonstrate that oral contraceptive steroids are relatively strong promoters of hepatocarcinogenesis and have little if any genotoxic effect.

Introduction

Previous reports have indicated that women who are long-term users of oral contraceptive steroids (OCS) are at increased risk of developing liver adenomas and hepatocellular carcinomas (*1, 2*). The data on the carcinogenicity of OCS in animals and humans, together with reports that such synthetic estrogens are not mutagenic in bacterial or mammalian cells suggest that they might be promoters of hepatocarcinogenesis (*1-4*).

Recent studies reported by Taper (*5*), Cameron et al. (*6*) and our laboratory (*1*) employing experimental animal model systems have demonstrated that various OCS, particularly estrogens, can promote chemical carcinogen-initiated hepatocarcinogenesis. Taper (*5*), using ovariectomized female Wistar rats, demonstrated that chronic treatment with estradiol-17-phenylproprionate and estradiol benzoate, following initiation with *N*-nitrosomorpholine, enhanced the appearance of liver foci, nodules and hepatocellular carcinomas. More recently, Cameron et al. (*6*) reported that feeding ethinyl estradiol to diethylnitrosamine initiated male Fischer 344 rats enhanced the appearance of γ-glutamyl transpeptidase-positive foci (putative preneoplastic lesions) and hyperplastic nodules.

This paper presents a brief review of our previously published work on the promoting (*1, 2*) and genotoxic (*2, 7*) properties of oral contraceptive steroids. In addition, data on the effects of these agents on the glucocorticoid response of cultured hepatoma cells and on the metabolic cooperation in V-79 Chinese hamster cells (*8*) is reported.

Materials and Methods

Female Sprague-Dawley rats (Charles River Breeding Laboratories, Inc., Wilmington, MA) were housed individually in wire bottom cages under controlled conditions of temperature, humidity and lighting. All animal diets were semipurified and obtained from Teklad (Madison, WI). Mestranol (M), norethynodrel (N) and phenobarbital (PB) were mixed into the diet and fed as previously reported (*1*).

For the promotion studies, at 7 weeks of age the animals were subjected to a partial hepatectomy followed, 24 hr later, by treatment with diethylnitro-

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samine (DEN) at 5 mg/kg body weight, or H_2O. At 24 hr thereafter, the animals were transferred to various treatment groups: (1) DEN → M; (2) DEN → N; (3) DEN → M + N; (4) H_2O → M + N; (5) DEN → PB; (6) H_2O → PB; and (7) DEN → basal diet. The OCS were fed at a dose level equivalent to 10 to 15 times the human level (*1*) which was for M, 0.02-0.03 mg/kg and for N, 0.5-0.75 mg/kg body weight/day. Phenobarbital was present at 0.05% in the diet which provides 19.4 to 27.4 mg/kg body weight/day. The animals were fed the various diets for 4 and 9 months (only the 9 month data are presented here). At the time of killing, the livers were processed for determination of the number of γ-glutamyl transpeptidase (GGT)-positive foci and histopathologic evaluation as previously reported (*1*).

Studies on the effects of mestranol and norethynodrel on metabolic cooperation were carried out by Dr. J. E. Trosko as described by Yotti et al. (*9*). Analysis of the initiating and genotoxic potential of various OCS were carried out *in vivo* and in primary cultures of rat hepatocytes and described by Yager and Fifield (*7*). Studies on the effects of M and N on the induction of tyrosine amino transferase (TAT) were done by using H-35 hepatoma cells provided by Ms. Joyce Becker (McArdle Laboratory for Cancer Research, U. Wisconsin, Madison, WI). The H-35 hepatoma cells were cultured in S-77 medium + 5% fetal calf serum, 20% horse serum and 4 *mM* glutamine. After 2 days of culture to near confluency in 6 cm dishes, the medium was changed to serum-free and the experiments were conducted 24 hr later. Test compounds were dissolved in ethanol, and TAT activity was determined as described by Butcher et al. (*10*) using the method of Diamondstone (*11*). One unit of activity is equal to 1 μmole of *p*-hydroxyphenylpyruvic acid formed/mg soluble protein/hour. Protein was determined by the method of Hartree (*12*).

Results and Discussion

In a previous paper (*1*), we reported that two oral contraceptive steroids, particularly mestranol, enhanced the appearance of GGT-positive putative preneoplastic lesions in the livers of DEN-initiated female Sprague-Dawley rats after both 4 and 9 months of treatment. Body weights were significantly lower in all groups ingesting M and/or N throughout the course of the experiment. At 9 months, liver weights per 100 g body weight were significantly greater in the groups receiving PB, M, N and M + N (*1*). The data in Table 1 were adapted from our previous report (*1*) and show the number of GGT-positive foci/cm² in the livers of animals from the various treatment groups. These data clearly show that M, M + N and the positive control, PB, significantly enhanced the number of GGT-positive foci. Norethynodrel alone caused a significant increase in GGT foci at 4 months (not shown) but not at 9 months. When one considers that M was present in the diet at approximately 0.125% the level of PB, it seems that M is a strong promoter, at least on a dose-for-dose basis.

It can also be seen from Table 1 that the number of GGT-positive foci was slightly but significantly elevated in the non-DEN-treated group fed M + N (H_2O → M + N). Recent results (Yager, unpublished observations) have demonstrated that feeding mestranol alone also results in a small but significant dose-dependent increase in GGT-positive foci. This is in contrast to PB, which does not cause an increase in such foci (Table 1). These results, together with other reports in the literature (*13, 14*) suggest that M, in addition to being a strong promoter, might have weak initiating potential and thus be capable of acting as a weak complete carcinogen (*7*).

Recently, we reported the results of studies aimed at determining whether mestranol has any initiating potential, and whether various OCS are genotoxic to hepatocytes (*7*). The results of these studies were negative. Using an initiation/promotion protocol, M had little, if any, ability to act as an initiator of the development of GGT-positive foci. In addition, neither M, N, nor ethinyl estradiol caused detectable levels of hepatocyte DNA damage demonstrating thus that they are not genotoxic agents (*7*). These results support the work of others who have failed to detect any ability by various synthetic estrogens to cause mutagenesis in the Ames assay (*3*), in mammalian cells (*4*) or chromosomal damage in rat bone marrow cells (*15*). Altogether, these results support the notion that the OCS are not genotoxic agents, whose effects are mediated via damage to DNA. Rather, these compounds may be strong promoters, acting on a spontaneous level

Table 1. GGT foci/cm² and percent rats of with GGT foci at 9 months.[a]

Group	GGT Foci/cm²[b]	Rats with foci, %
DEN→M	8.6 ± 2.4*,†	100
DEN→N	4.1 ± 2.7	60
DEN→M + N	7.3 ± 1.8†,**	100
H_2O→M + N	2.4 ± 0.8†	100
DEN→PB	6.4 ± 1.6†	100
H_2O→PB	0.2 ± 0.1	30
DEN→Basal	0.3 ± 0.1	60

[a] Adapted from Yager and Yager (*1*).
[b] Mean ± SE for ten animals/group.
* Significantly different from H_2O→M + N group, $p<0.01$.
† Significantly different from DEN→basal group, $p<0.001$.
** Significantly different from H_2O→M + N group, $p<0.005$.

of initiation in the non-DEN-initiated animals or on the latent initiated cells in DEN-treated animals. Additional experiments were conducted to explore some of the types of effects these agents exert on mammalian cells.

Phenobarbital, various OCS, progesterone and many other compounds are growth stimulatory for liver (*16, 17*). Loeb (*18*) has shown that corticosteroids inhibit DNA synthesis in liver cells. Desser-Wiest has reported that progesterone stimulates DNA synthesis and mitosis in liver and in cultured hepatoma cells, and that progesterone can prevent the liver cell growth inhibitory effects of corticosterone (*17, 19*). Thus, Desser-Wiest (*17*) has proposed a model by which progesterone can stimulate liver growth through inactivation of the corticosteroid receptors. Since some OCS are progesterone analogs, and since estradiol has been reported to be able to inhibit the energy-dependent uptake of corticosterone in isolated hepatocytes (*20*), we decided to determine whether M, N or M + N can inhibit the effects of hydrocortisone.

Glucocorticoid hormones induce TAT in liver cells and cells derived from hepatomas, including H-35 cells (*10*). The data in Figure 1 show that TAT activity increases approximately twofold 6 hr following treatment with 10^{-6} *M* hydrocortisone (HC). The induction of TAT by hydrocortisone was inhibited in a dose dependent manner by the simultaneous presence of progesterone, supporting the Desser-Wiest model (*17*) that progesterone interferes with glucocorticoid effects. In contrast, N did not inhibit the induction of TAT by HC, except at 10^{-4} *M*, where toxicity to the cells was evident. On the other hand, M did appear to have some inhibitory effect at 10^{-5} *M*, but not at the lower concentrations where progesterone was still significantly effective. These results suggest that the effect on liver growth by these two OCS is probably not mediated through inhibition of the growth inhibitory effects of corticosteroids by receptor inactivation as postulated for progesterone (*17*).

Of the many effects of tumor promoters on cells, their ability to inhibit metabolic cooperation, a measure of cell-cell communication, has recently received considerable attention (*2, 9, 21-25*). If growth regulatory information is exchanged between cells through gap junctions which mediate cell-cell communication, perhaps the inhibition of this process would create an environment where initiated cells, subtly altered in their sensitivity to growth regulatory factors, can begin to grow (*25, 26*). Together with Dr. Trosko, preliminary studies have been carried out to determine the effect of M, N and ethinyl estradiol on metabolic cooperation in V-79 Chinese hamster cells (*8, 9*). The results in

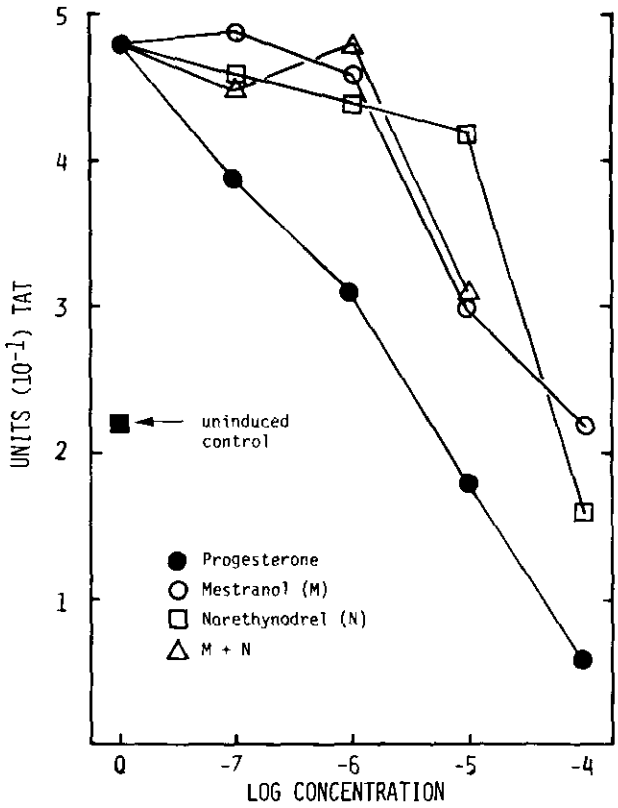

FIGURE 1. Effect of progesterone, mestranol and norethynodrel on the induction of TAT by hydrocortisone. H-35 hepatoma cells were cultured in S-77 medium supplemented with 5% fetal calf serum, 20% horse serum and 4 *mM* glutamine. At 24 hr prior to hormone addition, the cells were transferred to serum free medium. Hydrocortisone was added at 10^{-6}*M*, and the other hormones were added 30 min prior to hydrocortisone addition. The cells were harvested for TAT determination 6 hr later. One unit of TAT activity equals 1 μmole of *p*-hydroxyphenylpyruvic acid formed/mg soluble protein/hr.

Table 2. Effects of various OCS on metabolic cooperation.[a]

Treatment	Dose, *M*	Recovery of 6T6^r-V79 cells, %
–	–	48
TPA[b]	10^{-7}	117
Mestranol	2.5×10^{-6}	61
Ethinyl estradiol	2.5×10^{-6}	66
Norethynodrel	2.5×10^{-6}	85

[a] The assay was carried out as described by Yotti et al. (*9*).
[b] 12-O-tetradecanoylphorbol-13-acetate.

Table 2 indicate that these three OCS significantly inhibit metabolic cooperation in this cell culture system (*8*). While more detailed dose-response studies are required, these results suggest that the role of inhibition of cell-cell communication in promotion of hepatocarcinogenesis by the OCS needs to be investigated.

Conclusions

The results of our own work, together with those of others, indicate that various oral contraceptive steroids, particularly the synthetic estrogens, are promoters of hepatocarcinogenesis. The lack of detectable genotoxic and mutagenic effects suggests that they are not complete carcinogens, but rather compounds that modify and enhance the carcinogenic process through as yet unknown mechanisms. While these compounds could be classified as acting through epigenetic mechanisms as defined by Weisburger and Williams (*20*), we would prefer not to use that term and simply say that their mode of action appears to involve mechanisms other than genotoxic and mutagenic effects. Much additional study is required to determine the mechanism(s) of action of these agents in particular, and promoters in general.

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