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Enhancement in Rats by The Liver Tumor Promoter Ethinyl Estradiol of a Serum Factor(s) Which Is Stimulatory for Hepatocyte DNA Synthesis

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SUMMARY: Fractionation of female rat serum or plasma on Sephadex G-200 revealed the presence of an activity stimulatory for hepatocyte DNA synthesis. Treatment of female rats with the liver tumor promoter ethinyl estradiol (EE) at 2.5μg/day caused a 1.6 fold increase in the level of this activity at 24 hr in both serum and plasma. The stimulatory activity had a molecular weight of 135 kD, was sensitive to trypsin and heating and was not inhibited by the antiestrogen tamoxifen or antibody to epidermal growth factor (EGF). However, the pooled active fractions from EE-treated rats competed to a greater extent than comparable fractions from control rats for specific [^{125}I]-EGF binding to rat liver membranes. These results demonstrate that treatment of female rats with EE, under conditions known to stimulate liver growth, caused an increase in the level of a factor(s) stimulatory for hepatocyte DNA synthesis and whose activity may be mediated through the EGF receptor.

Results from our laboratory (1,2) and several others (3-5) have established that the synthetic estrogens mestranol and EE are strong promoters of hepatocarcinogenesis. Chronic EE treatment of female rats caused an increase in liver DNA synthesis which reached a maximum level by 24 hrs and persisted through 7 days (6). By 14 days DNA synthesis had returned to control levels where it remained at 21 days even with continuous EE treatment. Others have also demonstrated that EE and other steroid hormones transiently stimulate liver growth, mainly through hepatocyte hyperplasia (7). However, it is not known whether the liver growth stimulatory effects of EE are due to its direct or indirect effects on hepatocytes.

Several laboratories have detected and characterized factors stimulatory for hepatocyte DNA synthesis in the sera of partially hepatectomized rats (8-12). The goal of our study was to determine whether the level of such a factor(s), present in serum and/or plasma, is enhanced by treatment with EE. Our results show that EE enhanced the level of a serum/plasma activity stimulatory for DNA synthesis in primary cultures of rat hepatocytes. Furthermore, the factor(s) responsible for this stimulatory activity may be in the EGF/TGFα family.

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Abbreviations used: EE, ethinyl estradiol; EGF, epidermal growth factor; TGF, transforming growth factor; DMEM, Dulbecco's modified Eagle's medium; ITS, insulin, transferrin, selenium; PBS, NaCl, 0.14M; KH_2P0_4, 1.5mM; KCl, 3mM; Na_2HPO_4, 8 mM, pH 7.4; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; EGTA, ethyleneglycol-bis-(β-aminoethyl ether)N,N'-tetraacetic acid.

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MATERIALS AND METHODS

Animals: Female Lewis rats (Charles River Breeding Laboratories, Inc., Wilmington, MA.) were housed under controled conditions of temperature, humidity and light and received food and water ad libitum as described previously (6). All perfusions were performed at the end of the dark period.

Special Materials: Collagenase type I was obtained from Cooper Biomedical, Malvern, PA. The media, Ham's F-12 and DMEM (phenol red-free) were purchased from Gibco Laboratories, Grand Island, NY. The ITS preparation, EGF and EGF antiserum were from Collaborative Research Inc., Bedford, MA. EE and tamoxifen were purchased from Sigma Chemical Company, St, Louis, MO. [^{3}H]-Thymidine and [^{125}I]-NaI were from Dupont New England Nuclear, Boston, MA.

Animal Treatment: Treatment with ethinyl estradiol was performed by s.c. implantation of time-release tablets (2.5μg/day) purchased from Innovative Research of America (Toledo, OH).

Hepatocyte Isolation: Hepatocytes were isolated from female Lewis rats weighing between 130 and 180 g using a modification of the 2-step collagenase perfusion technique of Seglen (13) as described previously (14). The isolated hepatocytes were inoculated into collagen-coated culture dishes at a density of 2.4 x 10^4 cells/cm^2 in serum-free medium which consisted of a 1:1 mixture of Ham's F-12 and DMEM supplemented with ITS (insulin, 5μg/ml; transferrin, 5μg/ml; selenium, 5ng/ml). The cultures were maintained at 37^{0}C in a 5% $C0_2$ atmosphere. The medium was changed 4 hr later (time zero). The serum fractions and various agents being tested were added at time zero and present for 48 hr. In all experiments, hepatocytes were harvested at 48 hr.

[^{3}H]-Thymidine incorporation: The cells were pulsed with [^{3}H]-thymidine, 3-4μCi/culture, for 2 hr prior to harvest. Radioactivity in DNA, extracted with hot trichloroacetic acid (6) was determined using a Beckman 7000 liquid scintillation counter (Beckman Instruments Inc., Palo Alto, Ca). The incorporation of [^{3}H]-thymidine into DNA was expressed as dpm/mg cellular protein. Protein content was determined by the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, Ca).

Preparation of rat serum and plasma: Rat blood was collected from the descending aorta of rats under ether anesthesia. Blood from 5 rats was pooled and allowed to clot for 1 hr on ice, centrifuged at 1500*g* to remove the clot and the serum collected and stored at -70^{0}C until use. For the preparation of plasma, blood from 5 rats was drawn into chilled plastic syringes containing 3.8% sodium citrate, and centrifuged at 3000*g* to remove the cells. The blood was then recalcified by addition of $CaCl_2$ to a final concentration of 50 mM, allowed to clot overnight, and centrifuged at 1500*g*. The resultant plasma was collected and stored at -70^{0}C until use.

Gel filtration: For chromatography on Sephadex G-200 (Pharmacia Fine Chemicals, Uppsala, Sweden), 12ml serum or plasma was applied to a 3x65cm column that had been equilibrated with PBS. The serum or plasma was eluted with PBS at a flow rate of 20 ml/h. Seven to eight ml of eluate were collected in each fraction. Corresponding serum or plasma fractions from control and EE-treated rats were adjusted to the same protein concentrations as determined by the Bio-Rad protein assay. Each fraction was sterilized by filtration through a 0.2μm membrane and stored at -70^{0}C until use. The G-200 column was calibrated using a molecular weight standard mixture (Sigma) containing beta-amylase (200 kD), alcohol dehydrogenase (150 kD), albumin, (66 kD), carbonic anhydrase (29 kD), and cytochrome C (12.4 kD).

Characterization of the pooled serum fractions: Serum fractions corresponding to the peak of activity were pooled and dialyzed (M_r 6,000 cutoff) against medium overnight at 4^{0}C. Aliquots of the pooled serum fractions were heated in boiling water for 90 sec, at 50^{0}C for 30 min, or at 37^{0}C for 2 or 4 hrs. To test trypsin sensitivity, aliquots of the pooled serum fractions were incubated with trypsin (15μg/ml) for 4 hr at 37^{0}C; the reaction was terminated by addition of soybean trypsin inhibitor (30μg/ml). These samples were then sterilized by filtration through a 0.2 μm membrane. To test the effect of tamoxifen on the pooled serum fractions, tamoxifen was dissolved in 70% ethanol and then diluted to 20% ethanol with medium. After addition of tamoxifen to the cultures, the final ethanol concentration was 0.1%. Controls were exposed to the same concentration of ethanol. The EGF antiserum was diluted with medium and added to the cultures together with EGF or pooled active serum fractions.

EGF receptor assay: Rat liver membranes were isolated as described by others (15). Iodination of EGF was performed by the chloramine-T method (16). [^{125}I]-EGF binding to liver membranes was performed as previously described (17) with some modifications. Briefly, 40μg of liver membrane protein was incubated with [^{125}I]-EGF (2ng/ml) in 0.15 ml of binding buffer (20mM HEPES, 1.5mM EGTA and 0.1% bovine serum albumin) in the presence or absence of pooled serum fractions. The binding reaction continued for 40 min at room temperature and was stopped

by filtration through a Millipore GFC filter (Millipore Corporation, Bedford, MA). After extensive washing with binding buffer, the radioactivity on the filter was determined using a gamma-counter. Nonspecific binding was determined by incubation of liver membranes with [^{125}I]-EGF in the presence of 300-fold excess cold EGF.

RESULTS

Initial attempts to use unfractionated serum to detect the presence of a factor(s) stimulatory to DNA synthesis in hepatocytes in primary culture were unsuccessful due to large interanimal variations. Similar variability among serum samples prepared from individiual partially hepatectomized rats has also been reported by Nakamura et al. (10). Thus, we conducted experiments to determine whether enhanced levels of stimulatory factor(s) could be detected upon fractionation of serum from rats 24 hr after EE treatment. Fractionation of sera from control and EE-treated rats on Sephadex G-200 revealed two peaks of activity stimulatory for DNA synthesis (Fig. 1). The greatest activity was in the second peak which eluted at a volume corresponding to a M_r of 135 kD. The activity in sera from EE-treated rats eluted in the same position as that from control rats but was 1.6 fold higher at the peak.

Since much of the mitogenic activity of serum is due to soluble factors released from platelets during blood clot formation (18), it was possible that the difference in hepatocyte DNA synthetic stimulatory activity in sera from control and EE-treated rats was due to different concentrations of some stimulatory factors released from platelets. To investigate this possibility, plasma was collected from control rats and rats 24 hrs after EE tablet implantation and fractionated on Sephadex G-200. The hepatocyte DNA synthetic stimulatory activity in plasma had a fractionation pattern similar to that of sera and also showed a 1.5-fold increase upon EE treatment (data not shown).

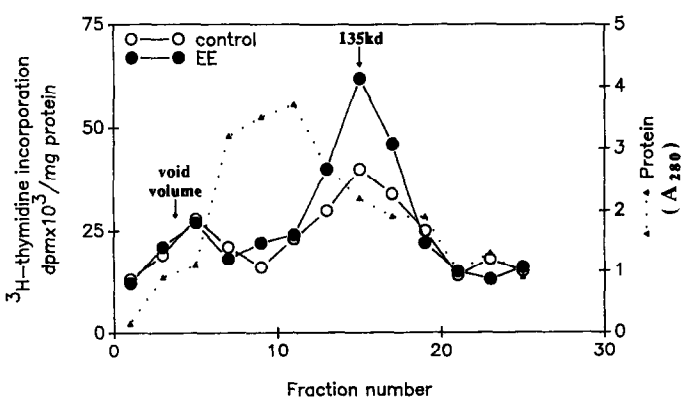


Figure 1 Fractionation of sera from control rats and rats 24 hr following EE treatment. Sera, pooled from 5 rats, was fractionated on Sephadex G-200 as described in "Materials and Methods". Corresponding serum fractions from control and EE-treated rats were equalized for protein content and added to the cultures at 25% by volume. DNA synthesis was measured by a 2 hr pulse with [^{3}H]-thymidine at 46-48 hr. Each point represents the value for a single culture. Similar results were detected in 5 separate experiments.

TABLE 1

EFFECTS OF VARIOUS TREATMENTS ON THE POOLED ACTIVE SERUM FRACTIONS FROM EE-TREATED RATS

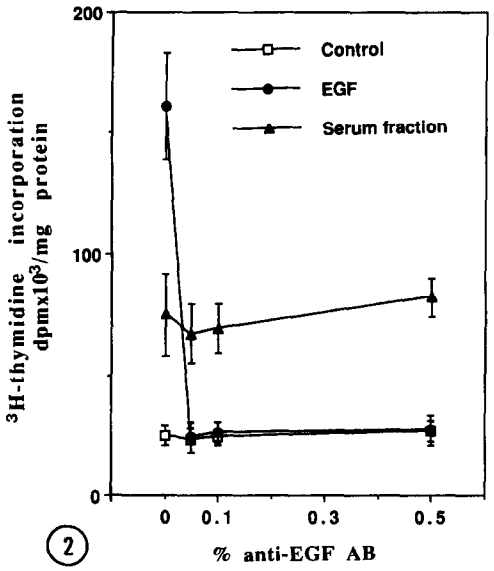
Treatment[a]	DNA Synthesis dpmx10^3/mg protein		Residual activity (%)	
	exp 1	exp 2	exp 1	exp 2
none	25± 2[b]	33± 3	--	--
untreated pool fractions	145±11	179±10	100	100
tamoxifen (1μM)	132±11	170± 8	91	95
37°C, 2 hr	178±15	240±19	123	134
37°C, 4 hr	--	181± 9	--	100
50°C, 30 min	107±15	110±10	74	61
100°C, 90 sec	29± 4	--	3	--
trypsin (37°C, 4 hr)	70± 9	76± 6	36	29

[a]Details of the treatments are described in "Materials and Methods". Sera pooled from 5 rats treated with EE for 24 hr was fractionated and assayed for stimulation of hepatocyte DNA synthesis as described in Fig. 1. The active fractions (14-16) were pooled, dialyzed against medium, and subjected to the different treatments indicated. For assay of DNA synthesis, pooled serum fractions were added to cultures at 50% by volume and present for 48 hr. [^{3}H]-Thymidine incorporation was measured at 48 hr following a 2 hr pulse. The data are from 2 separate experiments. The protein concentrations of pooled serum fractions in experiment 1 and 2 were 2.3mg/ml and 2.7mg/ml respectively.

[b]Values are mean ± the range of duplicate cultures.

In studies to be reported in detail elsewhere (Shi and Yager, submitted), we demonstrated that direct addition of EE to cultured hepatocytes caused a small 2-2.7 fold increase in DNA synthesis and that pretreatment of hepatocytes with EE greatly enhanced their DNA synthetic response to EGF (19). Thus, we investigated whether the increased hepatocyte DNA synthetic stimulatory activity in sera from EE-treated rats was due to EE itself, perhaps bound to a serum carrier protein and not separated during gel filtration. This was done by determinating the effect of the anti-estrogen, tamoxifen, on the stimulatory activity of pooled, active serum fractions from EE-treated rats. Tamoxifen, at 1μM, which blocked the ability of EE to stimulate hepatocyte DNA synthesis (19), was added to the cultures containing the pooled fractions. The results (Table 1) show that tamoxifen failed to inhibit the stimulation of hepatocyte DNA synthesis suggesting that the increased stimulatory activity in EE-treated rats was not due to the presence of EE in the serum fractions.

To partially characterize this activity, in subsquent experiments, serum fractions constituting the peak of stimulatory activity from EE-treated rats were pooled, subjected to various treatments and assayed for their ability to stimulate hepatocyte DNA synthesis. Two separate experiments using pooled serum fractions from different EE-treated (24 hr) rats were performed. As shown in Table 1, untreated pooled serum fractions from EE-treated rats, dialyzed against medium and diluted to 50% by volume with medium, increased [^{3}H]-thymidine incorporation more than 5-fold over control values. To test for heat sensitivity, aliquots of pooled fractions were



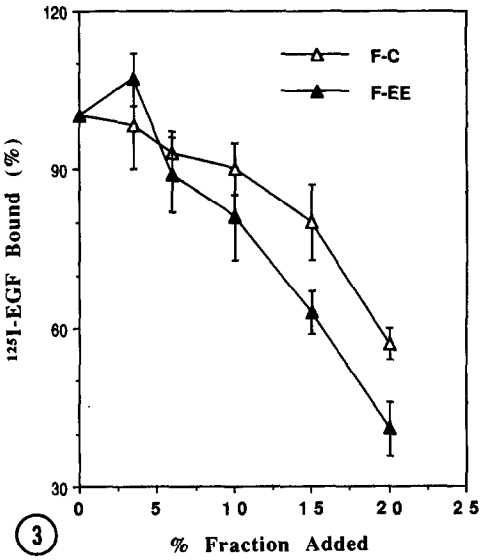


Figure 2 Effect of EGF antiserum on the stimulation of hepatocyte DNA synthesis by EGF and pooled active serum fractions from EE-treated rats. Active serum fractions (14-16) were pooled and dialyzed as described in "Materials and Methods". EGF (25ng/ml) and the pooled serum fractions (30% by volume) were present for 48 hr in the absence or presence of three different concentrations of EGF antiserum. DNA synthesis was assayed by measuring [^{3}H]-thymidine incorporation at 46-48 hr. Each point represents the mean ± the range of duplicate cultures.

Figure 3 Inhibition of [^{125}I]-EGF binding to rat liver membranes by pooled active serum fractions from control and EE-treated rats. Conditions for the EGF receptor assay were described in "Materials and Methods". The pooled active serum fractions from control and EE-treated rats were equalized for protein content (2.7mg/ml) and added to the reaction at 3, 6, 10, 15 and 20% by volume. The specific [^{125}I]-EGF binding in the absence of added serum fractions was considered as maximum (100%). Nonspecific binding was 12%. Each value represents the mean ± SD of four determinations from two separate experiments.

heated as follows: 37^{0}C for 2 or 4 hr, 50^{0}C for 30 min and 100^{0}C for 90 sec. No precipitation was observed under any of the test conditions. The stimulatory activity was lost completely upon heating at 100^{0}C. However, a slight, but reproducible increase in the activity was found after 2 hr at 37^{0}C. Exposure of the pooled fractions to trypsin substantially decreased the activity. These results demonstrate that this stimulatory activity is heat and trypsin sensitive.

Next, we explored the possible relationship between this activity and EGF by first testing the effect of EGF antiserum on the stimulatory activity. Hepatocyte DNA synthesis in response to pooled active serum fractions from EE-treated rats was assayed in the absence or presence of several dilutions of antiserum to EGF. The results are shown in Fig. 2. Hepatocytes were incubated with either EGF (25ng/ml) or pooled active serum fractions added to 30% by volume in the absence or presence of EGF antiserum at concentrations of 0.02%, 0.1% and 0.5% by volume. The stimulatory activity of EGF was completely inhibited by EGF antiserum at all three concentrations. However, EGF antiserum failed to supress the stimulatory activity of the active serum fractions.

The possible relationship between the serum stimulatory activity and growth factors in EGF/TGFα family was investigated further by determining whether the factor(s) could compete

with specific [^{125}I]-EGF binding to isolated rat liver membranes. [^{125}I]-EGF binding to liver membranes was performed in the absence or presence of several concentrations of pooled active serum fractions. As shown in Fig. 3, at equal protein concentrations, both pooled serum fractions from control and EE-treated rats exerted a dose-dependent inhibition of [^{125}I]-EGF binding to rat liver membranes. However, the pooled serum fractions from EE-treated rats showed a reproducibly greater inhibitory effect than that from control rats. As a control, non-stimulatory serum fractions eluted from the G-200 column were pooled and [^{125}I]-EGF binding to liver membranes was performed in the presence of this pooled fraction added at 5%, 10% and 15% by volume. No significant inhibition of [^{125}I]-EGF binding was observed (data not shown).

DISCUSSION

The ability to induce cell growth is a property shared by many promoters that is central to their promoting activity (20). Several studies have implicated a role for estrogen in liver regeneration induced by partial hepatectomy (21,22) and other studies have demonstrated the induction of liver growth by estrogens (6,7). However, none of these reports provided information pertaining to whether the hyperplastic response induced by estrogens occurred through direct or indirect effects on hepatocytes.

In primary cultures of rat hepatocytes, we have recently shown that EE alone enhanced DNA synthesis 2 fold over control; however, EE pretreatment followed by exposure to EGF caused a dramatic increase in the hepatocyte DNA synthetic response to EGF (19, and Shi and Yager, submitted). We have also demonstrated that this EE-induced increase in hepatocyte DNA synthetic response to EGF was associated with an increase in EGF receptor levels in intact cultured hepatocytes and on liver membranes isolated from rats treated with EE. In the present study, we demonstrated that EE can also exert an indirect stimulatory effect on hepatocyte DNA synthesis through stimulation of an increase in the level of a serum/plasma factor(s) stimulatory for DNA synthesis in cultured hepatocytes. Therefore, we hypothesize that liver growth stimulation by EE involves both direct and indirect effects on hepatocytes. On the one hand, EE treatment enhances the response of hepatocytes to EGF; on the other hand, EE also increases a serum/plasma hepatocyte growth factor(s), which may be in the EGF/TGFα family. Conformation of this hypothesis will require further study.

Results from several laboratories have demonstrated that stimulation of liver growth is associated with increased levels of hepatocyte growth factor(s) in serum (8-12, 23-26). Michalopoulos, *et al.* (8,9) identified two serum hepatocyte growth factors named HPTA and HPTB, from partially hepatectomized rats. HPTA, with a molecular weight of about 120 kd, is similar to the factor identifized in our system with respected to size and sensitivity to heat. Russell and coworkers (27,28) reported that rat platelets contain a hepatocyte growth factor and suggested that the factor in serum is derived from platelets. In addition, Nakamura et al. (10) characterized a serum hepatocyte growth factor, "hepatotropin", from partially hepatectomized rats. Recently, the same group purified a hepatocyte growth factor from rat platelets and suggested that this platelet derived hepatocyte growth factor was very similar to the hepatotropin identified in serum (11, 12).

At the present time, we do not know the source of the hepatocyte growth factor(s) whose level is enhanced by EE. Although the factor(s) shows competitive inhibition of [^{125}I]-EGF binding to liver membranes, it is possible that the activities that competed with [^{125}I]-EGF binding and that stimulated hepatocyte DNA synthesis are from different factors. Our data do not rule out the existence of several hepatocyte stimulatory factors in the crude serum fractions.

The effects of estrogen on blood coagulation have been extensivly investigated (29,30). EE affects blood clotting by increasing the activity of coagulation factors, enhancing platelet activity, and decreasing antithrombin III (31). It is possible that the EE-induced increased serum hepatocyte stimulatory activity is derived from platelets due to its effects on them. Further purification is required to determine whether this factor is similar to the hepatocyte growth factor(s) purified from rat platelets by Nakamura et al.(11, 12) or from serum by Michalopoulos (8,9).

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