The Effect of Estrogen on Hepatocytes in Primary Culture

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INTRODUCTION

A variety of hormones and other growth factors have been shown to affect hepatocyte proliferation both in vivo and in vitro. We have attempted to identify and purify hepatic mitogens by investigating the requirements for DNA synthesis of hepatocytes in primary culture.

Epidermal growth factor appears to play an important role in the regulation of hepatocyte proliferation in primary culture. In the last few years there have been several reports on replicative DNA synthesis of hepatocytes in primary culture (8,13,15,19,22, 25,29). These reports agree that newly isolated hepatocytes can be maintained for several days in serum-supplemented or serum-free medium and can be induced to synthesize DNA if certain hormones or other growth factors are provided (3,5,7-10,13,16,20, 21,23,29,31). In general, EGF seems to be an essential hormone for replicative DNA synthesis in primary cultures of hepatocytes (11). Other hormones and/or growth factors have been shown to enhance or reduce the EGF effect (3,12-15,17,26,31,32).

The sex of the animals from which hepatocytes have been isolated has received little attention or has not been specifically addressed. It is well known that there are differences in liver function between male and female rats (2,5,6,24,28). In addition there is some recent evidence suggesting that sex hormones may play a modulator role in liver regeneration (9,10).

By testing the effects of estrogen on EGF-induced DNA synthesis of hepatocytes in primary culture we have found evidence that estrogen modifies the EGF effect.

MATERIALS AND METHODS

Male and female Fischer (F344) rats were obtained from Hilltop Lab Animals, Inc., Scottdale, PA. The animals were kept in a temperature and a light controlled room and received food and water ad libitum. Partial hepatectomy and sham hepatectomy were performed according to Higgins and Anderson (14) and all operations were performed between 7:30 and 9:00 a.m. The source of hormones and chemicals, the isolation of hepatocytes and the determination of (^{3}H)thymidine incorporation *in vitro* have been described (11).

For *in vivo* DNA synthesis male or female rats had 70% of their livers removed between 7:00 and 9:00 a.m. One hour before the indicated times, the animals were injected with 10 μCi (^{3}H)thymidine intraperitoneally. Animals were killed by cervical dislocation, the livers rapidly removed and frozen. Citric acid nuclei were prepared for (^{3}H)thymidine incorporation (4).

For EGF binding studies, cells were incubated for 3 hours in attachment medium and for an additional 24 hours in basal MEM plus insulin. The washing with binding medium and a 1-hour incubation at 4° in the presence of 0.125 to 16 nmol ^{125}I-EGF was as previously described (12). Binding equilibrium is fully established at 45 min. Subsequent washing, extraction of bound ^{125}I-EGF and determination of radioactivity were as described (12). This treatment removes over 90% of the cell bound radioactivity, and it does not remove the iodine from the EGF (12). DNA determination on selected dishes from each hepatocyte preparation indicated no loss of cells by this treatment. Nonspecific binding was determined by measuring ^{125}I-EGF binding in the presence of excess unlabeled EGF (1 μg/ml) and was approximately 1% of the total binding. Protein was determined by the method of Lowry et al. (18). Equilibrium dissociation constants (K_ds) and the concentration of binding sites were calculated by the method of Scatchard (27). The unpaired Student's t-test was used for statistical analysis of the data.

RESULTS

We first became aware that sex-related steroid hormones might be important for proliferation of hepatocytes when we compared hepatocytes from male and female rats in primary culture. Cell viability varied between 90% and 98% and was not sex related. Protein and DNA content were measured in cultures maintained in MEM at 48 hours after seeding. There was no significant difference between cultures of male and female hepatocytes. Further-

more, cell cultures derived from both male and female animals consisted almost exclusively of hepatocytes as 98% of the cells stained for glucose-6-phosphatase (30) after 72 hours in culture.

The ability of the hepatocytes isolated from female rats to synthesize DNA in monolayer cultures, however, was very much reduced from that of similar cultures of male hepatocytes as shown in Table 1. With MEM alone or MEM plus insulin there was no difference in DNA synthesis, but when EGF was added to the culture medium the difference was pronounced.

TABLE 1. Incorporation of [^{3}H]thymidine into primary cultures of hepatocytes isolated from male and female rats

Medium additions	Male	Female
	[^{3}H]thymidine incorporation (cpm/μg DNA)[a]	
0	1630 ± 210[b]	1593 ± 116
Insulin	1555 ± 96	1673 ± 231
EGF	12556 ± 940	5617 ± 458
Insulin + EGF	19231 ± 1270	6195 ± 762

[a]Cultures were processed after 72 hours in culture for incorporation of [^{3}H]thymidine which had been added 24 hours earlier.
[b]Numbers are average for 9 determinations ± S.D.

TABLE 2. Incorporation of [^{3}H]thymidine into primary cultures of hepatocytes isolated from male and female rats 24 hours after 70% hepatectomy

Medium additions	Male	Female
	[^{3}H]thymidine incorporation (cpm/μg DNA)[a]	
0	4610 ± 398[b]	1672 ± 210
Insulin	5047 ± 617	1586 ± 136
EGF	27150 ± 3400	11419 ± 938
Insulin + EGF	36625 ± 2750	13618 ± 1220

[a]Assay conditions were as for Table 1.
[b]Numbers are averages of 6 determinations ± S.D.

Slightly different results were obtained when DNA synthesis was compared in vitro in hepatocytes isolated from male or female rats 24 hours after partial hepatectomy as shown in Table 2. In male hepatocytes from regenerating liver, regardless of incuba-

tion medium, DNA synthesis was increased 2-3 fold over that observed in hepatocytes from normal liver. In females there was also a 2-fold increase of DNA synthesis when hepatocytes from regenerating liver were incubated in the presence of EGF. In the absence of EGF, however, there was no increase. This resulted in the interesting finding that hepatocytes, isolated from male or female regenerating liver, responded to EGF with an equivalent fold increase, but not with the same magnitude as opposed to hepatocytes, isolated from non-hepatectomized rats, in which those isolated from males respond to EGF much better than those isolated from females. We have shown previously by autoradiography that (^{3}H)thymidine incorporation represents replicative DNA synthesis (11).

The possibility was considered that the lack of significant stimulation of DNA synthesis by EGF in hepatocytes isolated from female rats might be due to differences in the regenerative response between male and female rats in vivo. This does not seem to be the case as seen from the results in Table 3. On the contrary, the numbers obtained with female rats were more consistent and at least of the same if not higher magnitude. In addition, the labeling index determined at 28 hours after partial hepatectomy was the same, with 33.2% and 34.4% labeled cells in male and female rats, respectively.

Since the more dramatic differences in DNA synthesis between male and female hepatocytes were seen in the presence of EGF, we studied EGF binding to male and female hepatocytes in primary cultures.

The binding curve and Scatchard analysis are shown in the Figure 1. Female hepatocytes bound more EGF than male hepatocytes, B_{Max} 9.1 x 10^{-10}M vs. B_{Max} 2.8 x 10^{-10}M.

However, female hepatocytes exhibited significantly lower affinity for EGF (K_d 3.17 x 10^{-8}M) than did male hepatocytes (K_d 5 x 10^{-9}M).

EGF binding studies were also done on male and female hepatocytes isolated 24 hours after 70% hepatectomy. The Scatchard analysis is shown in Figure 2. In both cases only very low affinity binding is evident, K_d=1.37 x 10^{-7}M for male hepatocytes, and K_d=1.05 x 10^{-7}M for female hepatocytes. We than considered the possibility that estrogen might influence the mitogenic effect of EGF. The results in Figure 3 indicate that this is indeed the case. When estrogen at physiological concentrations was added to male hepatocytes during EGF binding studies, EGF binding to these male hepatocytes was similar to that found for female hepatocytes, as shown in Figure 1.

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TABLE 3. DNA synthesis in livers of male and female rats at different times after partial hepatectomy

Time after 70% hepatectomy	Male	Female
	[^{3}H]thymidine incorporation[a] (cpm/mg DNA)	
20 hours	4760 ± 670[b]	9890 ± 840
24 hours	24400 ± 5720	27520 ± 2300
28 hours	6990 ± 1250	5850 ± 310

[a]Animals were injected with [^{3}H]thymidine one hour before the indicated times.
[b]The numbers are from 6 animals ± S.D.

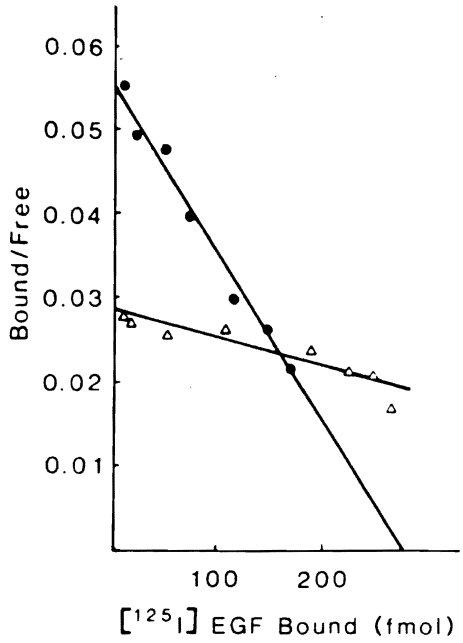

FIG. 1. EGF Binding: Scatchard analysis obtained from male and female hepatocytes. Culture and binding conditions are reported in "Materials and Methods." . ●-● , male hepatocytes: ▲-△, female hepatocytes.

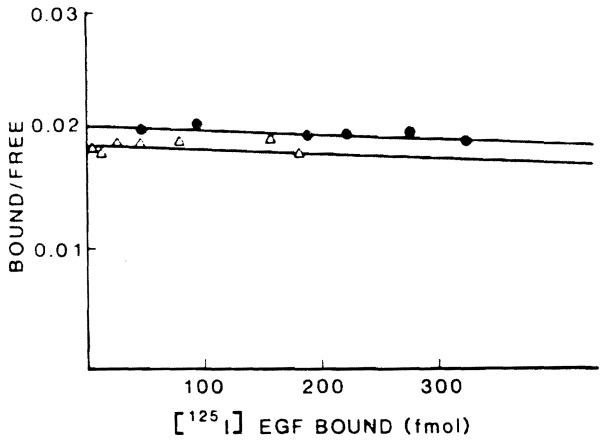

FIG. 2. EGF Binding: Scatchard analysis obtained from male Δ-Δ and female ● - ● hepatocytes prepared 24 hours after 70% hepatectomy.

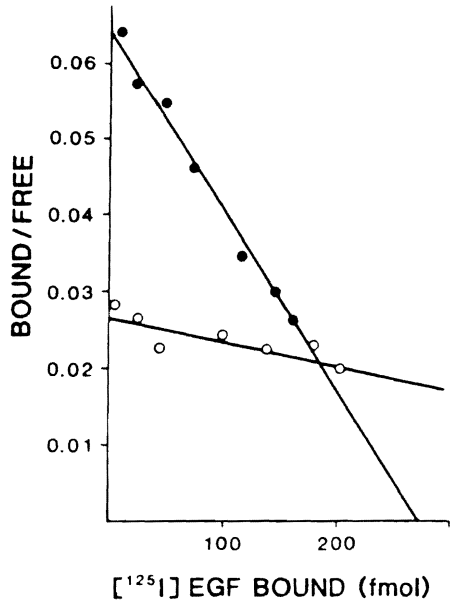

FIG. 3. EGF Binding: Scatchard analysis obtained from male hepatocytes incubated for one hour without ● - ●, and with O-O 10 pg/ml of estradiol.

These results become even more significant in view of the finding that estrogen inhibits EGF-induced (^{3}H)thymidine incorporation in hepatocytes as well, as shown in Table 4. Considerably higher levels of estrogen were needed to inhibit DNA synthesis than the levels required to inhibit EGF binding. The reason for

this might be that (^{3}H)thymidine incorporation is measured over a 24-hour period, present in culture between 24-48 hours, with estrogen and EGF present from the time of plating. During the binding studies both estrogen and EGF are present for only 1 hour. It is unlikely that the inhibition of (^{3}H)thymidine incorporation by estrogen is a non-specific effect associated with larger than physiological levels of steroids. The additions of testosterone, up to 10 times the estrogen levels had no effect on (^{3}H) thymidine incorporation.

The important aspect is that the addition of estrogen can change the EGF binding potential of male hepatocytes to that of female hepatocytes and that the addition of estrogen to male hepatocytes alters the magnitude of the EGF-induced (^{3}H)thymidine incorporation, approaching that of female hepatocytes.

TABLE 4. Estrogen inhibition of EGF-induced DNA synthesis in hepatocytes

Addition of estrogen (μg/ml)	Source of hepatocytes: Male — [^{3}H]thymidine incorporation[a] cpm/10^5 cells	Male — % inhibition	Female — cpm/10^5 cells	Female — % inhibition
0	8702 ± 1260[b]	0	5059 ± 402	0
5	5838 ± 812	23	3116 ± 610	38
10	4013 ± 220	54	1987 ± 96	61
20	3096 ± 160	64	1497 ± 187	70

[a] Cells were exposed to [^{3}H]thymidine from 3-27 hours after plating. Indicated amounts of estrogen were present during this time.

[b] The values shown are the averages of 6 determinations ± S.D.

DISCUSSION

Despite no apparent differences in the magnitude or onset of hepatic DNA synthesis in vivo in male and female rats following partial hepatectomy, EGF-stimulated DNA synthesis is dramatically different in male and female hepatocytes in primary culture. Female hepatocytes are indistinguishable from male hepatocytes with respect to morphological appearance, DNA content, protein content and (^{3}H)thymidine incorporation in the absence of EGF.

Female hepatocytes, on the other hand, respond to a much lesser extent than male hepatocytes, with increased DNA synthesis to the presence of EGF.

The results obtained with EGF binding studies provide a possible explanation for these findings. Binding studies performed using intact hepatocytes after 2 or 24 hours in primary culture indicated that hepatocytes derived from male animals had more high affinity EGF binding sites than hepatocytes derived from female rats. Female hepatocytes in culture show only EGF binding of very low affinity. More importantly, the data in Figure 3, using male hepatocytes in the presence of estrogen at physiological dosage, show EGF receptor behavior which is similar to that observed with female hepatocytes in the absence of hormones, and the data in Table 4 indicate that estrogen inhibits EGF-induced DNA synthesis in male hepatocytes. Male and female hepatocytes isolated 24 hours after partial hepatectomy did not exhibit the difference in EGF binding. As shown in Figure 2, with both cell preparations there was only low EGF binding affinity. Furthermore, in regenerating hepatocytes EGF induced the same degree of DNA synthesis in male and female hepatocytes. It appears from these data that the difference found between male and female hepatocytes isolated from normal liver depends only on the different levels of EGF receptors with high affinity. In fact, when EGF is added to hepatocytes which have the same EGF receptor state (regenerating hepatocytes) the percent of activation is the same.

In liver regeneration after hepatectomy there is no sex-related difference in vivo. Since EGF binding is different in male and female newly isolated hepatocytes, and also in membrane preparations (1,7), factors other than EGF seem to be responsible for in vivo regeneration.

One possibility is that the observed decrease in binding of EGF to female hepatocytes is due to the blocking of the receptor by estrogen, either through a direct competition for the receptor, or to an indirect mechanism. This interpretation would also make sense in view of the binding studies of EGF to male hepatocytes in the presence of estrogen. The addition of EGF to normal male hepatocytes brings about internalization of EGF-receptor complexes and thus down-regulation. Internalization and down-regulation does not occur to the same extent in female hepatocytes and in male hepatocytes if estrogen is added, because the EGF receptors are not accessible to EGF binding.

This hypothesis can also be applied to our findings in vivo following partial hepatectomy. If we suppose that partial hepa-

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4. Coetzee, M.L.,
Cancer Res., 4

5. Eacho, P.I.,
8:385-389.

6. Eagon, P.K.,
Van Thiel, D.H.

7. Earp, H.S.,
67:1580-1583.

8. Enat, R., Je
Leinwand, L.A.,
USA, 81:1411-14

9. Francavilla, A
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tectomy induces an internalization of EGF receptors by a mechanism not mediated by an EGF receptor complex formation but independent of EGF altogether, a similar down-regulation would occur in male and female rat liver following partial hepatectomy. Even though in the female rat the EGF receptors would be to a great extent blocked by estrogen, this might not prevent the internalization, since in this proposed model it would not be dependent on a ligand receptor interaction.

Another explanation is the possible existence of a mechanism activated after the hepatectomy which is able to change the EGF receptor state so that in male and female hepatocytes, the same receptor patterns are approached.

REFERENCES

1. Benveniste, R., and Carson, S.A. (1985): Mol. Cell. Endocrinol., 41:147-151.

2. Bone, S.N. III, Michalopoulos, G., and Jirtle, R.L. (1985): Cancer Res., 45:1922-1928.

3. Brown, K.D., Blay, J., Irvine, R.F., Helsop, J.P., and Berridge, M.U.U. (1984): Biochem. Biophys. Res. Commun., 123:377-384.

4. Coetzee, M.L., Short, J., Klein, K., and Ove, P. (1982): Cancer Res., 42:155-160.

5. Eacho, P.I., and Weiner, M. (1980): Drug Metab. Dispos., 8:385-389.

6. Eagon, P.K., Porter, L.E., Francavilla, A., Di Leo, A., and Van Thiel, D.H. (1985): Seminars in Liver Disease, 5:59-69.

7. Earp, H.S., and O'Keefe, E.J. (1981): J. Clin. Invest., 67:1580-1583.

8. Enat, R., Jefferson, D.M., Ruiz-Opazo, W., Gatmaitan, Z., Leinwand, L.A., and Reid, L.M. (1984): Proc. Natl. Acad. Sci. USA, 81:1411-1415.

9. Francavilla, A., Di Leo, A., Eagon, P.K., Wu, S-Q., Ove, P., Van Thiel, D.H., and Starzl, T.E. (1984): Gastroenterology, 86:552-557.

10. Francavilla, A., Eagon, P.K., Di Leo, A., Polimeno, L., Panella, C., Aquilino, A.M., Ingrosso, M., Van Thiel, D.H., and Starzl., T.E. (1986): Gastroenterology, 91:1263-1270.

11. Francavilla, A., Ove, P., Polimeno, L., Sciascia, C., Coetzee, M.L., and Starzl, T.E. (1986): Cancer Res., 46:1318-1323.

12. Haigler, H.J.T., Maxfield, F.R., Willingham, M.C., and Pastan, I. (1980): J. Biol. Chem., 225:1239-1241.

13. Hasegawa, K., Watanabe, K., and Koga, M. (1982): Biochem. Biophysis. Res. Commun., 104:259-265.

14. Higgins, G.M., and Anderson, R.M. (1931): Arch. Pathol., 12: 186-202.

15. Koch, K.S, and Leffert, H.L., (1979): Cell, 18:153-163.

16. Koch, K.S., Shapiro, P., Skelly, H., and Leffert, H.L. (1982): Biochem. Biophys. Res. Commun., 109:1054-1060.

17. Lin, Q., Blaisdell, J., O'Keefe, E., and Hearp, H.S. (1984): J. Cell. Physiol., 119:267-272.

18. Lowry, O.H., Rosebrough, N.Y., Farr, A.L., and Randall, R.J. (1951): J. Biol. Chem., 193:265-275.

19. McGowan, J.A., and Bucher, N.R.L. (1983): In Vitro, 19:159-166.

20. McGowan, J.A., Russell, W.E., and Bucher, N.R.L. (1984): Federation Proc., 43:131-133.

21. McGowan, J.A., Strain, A.J., and Bucher, N.R.L., (1981): J. Cell Physiol., 103:353-363.

22. Michalopoulos, G., Cianciulli, H.D., Novotny, A.R., Kligerman, A.D., Strom, S.C., and Jirtle, R.L. (1982): Cancer Res., 42:4673-4682.

23. Michalopoulos, G., Houck, K.A., Dolan, M.L., and Luetteke, N.C. (1984): Cancer Res., 44:4414-4419.

24. Norstedt, G
Skett, P. (1

25. Richman, R.
(1976): Proc

26. Rozengurt,
Chem., 256:

27. Scatchard,

28. Short, J.A
Zemel, R.,
1766.

29. Tomita, V.
Res., 135:3

30. Wachstein,
27:13-23.

31. Wharton, W
(1982): Pro

32. Wolffe, A.
Cell Endocr

24. Norstedt, G., Eneroth, P., Gustafsson, J., Hokfelt, T., and Skett, P. (1980): Brain Res., 192:77-88.

25. Richman, R.A., Claus, T.H., Pilkis, S.J., and Friedman, D.L. (1976): Proc. Natl. Acad. Sci. USA, 73:3589-3593.

26. Rozengurt, E., Brown, K.D., and Pittican, P. (1981): J.Biol. Chem., 256:716-722.

27. Scatchard, G. (1949): Ann. N.Y. Acad. Sci., 151:660-672.

28. Short, J.A., Brown, R.F., Husakova, A., Gilbertson, J., Zemel, R., and Lieberman, I. (1972): J.Biol. Chem., 247:1757-1766.

29. Tomita, V., Nakamura, T., and Ichihara, A. (1981): Exp. Cell Res., 135:363-371.

30. Wachstein, M., and Meisel, E. (1957): Am. J. Clin. Pathol., 27:13-23.

31. Wharton, W., Leof, E., Pledger, W.J., and O'Keefe, E.F. (1982): Proc. Natl. Acad. Sci. USA, 79:5567-5571.

32. Wolffe, A.P., Rakhmet, I.B., and Jamshed, R.T. (1985): Mol. Cell Endocrinol., 40:167-173.