GASTROENTEROLOGY 1987;93:597–605

695

Different Response to Epidermal Growth Factor of Hepatocytes in Cultures Isolated From Male or Female Rat Liver

Inhibitor Effect of Estrogen on Binding and Mitogenic Effect of Epidermal Growth Factor

ANTONIO FRANCAVILLA, PETER OVE, LORENZO POLIMENO, CONCETTA SCIASCIA, MONA COETZEE, RICCARDO PELLICI, SATORU TODO, IGAL KAM, and THOMAS E. STARZL

Department of Gastroenterology, University of Bari, Bari, Italy; Institute of Surgery, University of Torino, Torino, Italy; Department of Anatomy and Cell Biology and Department of Surgery, University Health Center of Pittsburgh, University of Pittsburgh and Veterans Administration Medical Center, Pittsburgh, Pennsylvania

Deoxyribonucleic acid (DNA) synthesis in hepatocytes isolated from the livers of male and female rats has been compared in monolayer culture. Plating efficiency, DNA and protein content, viability, and morphologic appearance were the same in cultures prepared with hepatocytes isolated from male or female rats. Epidermal growth factor (EGF)-induced DNA synthesis was significantly higher in hepatocytes from male rats than in hepatocytes from female rats. This was the case whether hepatocytes were isolated from normal or partially hepatectomized male or female rats. Hepatocytes isolated from regenerating liver synthesize more DNA than those isolated from normal liver in response to EGF. This increased response to EGF in hepatocytes derived from regenerating liver was relatively the same for male- and female-derived hepatocytes, but the magnitude of the response was considerably higher in male-derived hepatocytes. In contrast, in vivo DNA synthesis in the liver remnant after partial hepatectomy was similar in male and female rats if measured 24 h after the operation. A comparison of EGF binding to male- and female-derived hepatocytes maintained in primary culture indicated a lower number of high-affinity receptors for EGF in the female hepatocytes. The addition of estrogen to primary cultures of hepatocytes isolated from male rats inhibited EGF binding as well as EGF-induced DNA synthesis. Our studies show significant differences in DNA synthesis in response to EGF when male and female hepatocytes are compared in primary culture. The regenerative response after partial hepatectomy, on the other hand, was the same in male and female rats. Thus, our studies indicate that the sex of the donor rat is important when hepatocytes in culture are used for a variety of studies, such as hepatocyte metabolism, induction and control of DNA synthesis, and hepatocarcinogenesis. In addition, our results indicate that caution is advised when inferences are made from in vitro findings for in vivo conditions.

Epidermal growth factor (EGF) 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 deoxyribonucleic acid (DNA) synthesis of hepatocytes in primary culture (1–7). Most of 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

Received June 9, 1986. Accepted March 23, 1987.

Address requests for reprints to: Antonio Francavilla, M.D., Veterans Administration Hospital, Building 6, Circle Drive, Pittsburgh, Pennsylvania 15240.

This study was supported by a research project grant from the Veterans Administration, grant AM29961 from the National Institutes of Health, and by grant 885/02 16544 from Consiglio Nazionale delle Ricerche, Italy.

The authors thank Dr. Patricia Eagon and Dr. Alfredo DiLeo for advice on the binding studies and John Prelich for technical assistance.

© 1987 by the American Gastroenterological Association

0016-5085/87/$3.50

Abbreviations used in this paper: EGF, epidermal growth factor; FCS, fetal calf serum.

if certain hormones or other growth factors are provided (3,5,7–10). In general, EGF seems to be an essential hormone for replicative DNA synthesis in primary cultures of hepatocytes (11). Other hormones or growth factors, or both, have been shown to enhance or reduce the EGF effect (12–16).

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. Bone et al. (17) recently reported a dramatic difference in γ-glutamyltranspeptidase activity when male and female rats were compared. It has been shown that liver DNA synthesis can be stimulated in vivo by the injection of T_3, and female rats responded with a higher level of DNA synthesis than male rats (18). A crude membrane fraction from female rat liver has been shown to bind more prolactin than membrane fractions from male rats (19). The effect of diabetes on drug metabolism was also found to be different in male and female rats (20). Sexual dimorphism of hepatic enzymes and hormone receptors has recently been reviewed (21).

We have compared hepatocytes in monolayer cultures isolated from male and female Fischer F(344) rats (Hilltop Lab Animals, Inc., Scottdale, Pa.). When male and female rat hepatocytes were compared, we found dramatic differences in the ability of these hepatocytes to respond with replicative DNA synthesis in several different media conditions.

Materials and Methods

Animals

Male and female Fischer F(344) rats were obtained from Hilltop Lab Animals. The animals were kept in a temperature- and light-controlled room and received food and water ad libitum. Partial hepatectomy and sham hepatectomy were performed according to Higgins and Anderson (22), and all operations were performed between 7:30 and 9:00 AM.

Materials

Collagenase type I (125–250 U/mg) was obtained from Worthington Diagnostic Systems, Freehold, N.J. Eagles MEM and fetal calf serum (FCS) were purchased from GIBCO Laboratories, Grand Island, N.Y. Insulin, EGF, HEPES, and pyruvic acid were purchased from Sigma Chemical Company, St. Louis, Mo. [methyl-^{3}H]thymidine (50–80 Ci/mmol) and ^{125}I-EGF (150–200 μCi/μg) were obtained from New England Nuclear, Boston, Mass.

Isolation of Hepatocytes

Hepatocytes were isolated from 7-wk-old male or female rats weighing between 150 and 250 g by a modification of the in situ two-step collagenase perfusion technique of Seglen (23) as described by Jirtle et al. (24). The female rats weighed an average of 175 g and male rats of the same age weighed 200–250 g. The liver was perfused through a cannula in the inferior vena cava with 250 ml of buffer (142 mM NaCl, 6.7 mM KCl, 10 mM HEPES; pH = 7.4) followed by 250 ml of the same buffer containing in addition 5.7 mM $CaCl_2$ and 0.5 mg/ml collagenase. The hepatocytes were dispersed and washed twice with cold Ca^{2+}-free perfusion buffer and resuspended in basal medium (MEM) supplemented with pyruvate (1 mM), aspartate (0.2 mM), serine (0.2 mM), gentamycin (40 μg/ml), and for attachment insulin (10^{-7} M) and 5% FCS were added. Viability was determined by trypan blue exclusion (6) and only preparations having >90% viability were used. Cell number was determined with a hemocytometer.

The cells were plated at a cell density of 2×10^5 per 35-mm Falcon Primaria tissue culture dish in 1.5 ml of medium and maintained at 37°C in a 5% CO_2 atmosphere. After a 3-h attachment period, serum-free basal medium was added and hormonal additions were made as indicated. Epidermal growth factor and insulin, when added, were at a concentration of 10 ng/ml and 10^{-7} M, respectively.

[^{3}H]Thymidine Incorporation and Deoxyribonucleic Acid Determination

After the 3-h period to allow for attachment of the hepatocytes, the medium was changed and the appropriate additions were made. [^{3}H]Thymidine, 7.5 μCi per dish, was presesnt from 24–48 h or from 48–72 h. Each experimental group comprised four dishes. When the cells were harvested, one dish was used to determine DNA content using the microfluorometric method of Setaro and Morley (25), and the three remaining dishes of each group were treated as described by Michalopoulos et al. (26) to measure [^{3}H]thymidine incorporation.

Autoradiography

For autoradiographic studies, hepatocytes plated on Primaria dishes were fixed directly on the dishes. [^{3}H]Thymidine, 7.5 μCi per dish, was present for 24 h before fixation. Fixed cells in the dishes were covered with emulsion (Kodak NTB3, Eastman Kodak Co., Rochester, N.Y.) and the dishes were developed after 10 days. The labeling index was determined by counting a total of 1000 cells.

Determination of Deoxyribonucleic Acid Synthesis In Vivo

Male or female rats had 70% of their liver removed beween 7 and 9 AM. One hour before the indicated times, the animals were injected with 10 μCi [^{3}H]thymidine intraperitoneally. Animals were killed by cervical dislocation and the livers were rapidly removed and frozen. Citric acid nuclei were prepared for [^{3}H]thymidine and DNA determination (27). Microscope slides of formaldehyde-fixed liver samples were also prepared from male and female rats at 24-h after partial hepatectomy. The slides

were incubated in emulsion (Kodak NTB3) and developed for autoradiography.

Epidermal Growth Factor Binding Experiments

Epidermal growth factor binding studies were performed essentially as previously described (28). Cells were incubated for 3 h in attachment medium and for an additional 24 h in basal MEM plus insulin. Dishes were washed three times with binding medium (MEM plus insulin and 0.1% bovine serum albumin) and cooled to 4°C. Cells were then incubated in 1 ml of the binding medium in the presence of 0.125–16 nmol ^{125}I-EGF at 4°C for 1 h. After a 45-min incubation, binding equilibrium had been fully established. After the incubation, the dishes were washed five times with the binding medium and then treated for 6 min with 0.7 ml of 0.2 M acetic acid, pH 2.5, containing 0.5 M NaCl to remove cellular bound EGF. This treatment was repeated with 0.4 ml of acetic acid. It has previously been shown that this treatment removes more than 90% of the cell-bound radioactivity and it does not remove the iodine from the EGF (28). Deoxyribonucleic acid determination on selected dishes from each hepatocyte preparation indicated no loss of cells by this treatment. The acetic acid washes were combined and radioactive content was determined using a Packard Tricarb Liquid Scintillator (Packard Instrument Corp., Downers Grove, Ill.). Nonspecific binding was determined by measuring ^{125}I-EGF binding in the presence of excess unlabeled EGF (1 μg/ml) and was ~1% of the total binding. Protein was determined by the method of Lowry et al. (29). Equilibrium dissociation constants (K_d) and the concentration of binding sites were calculated by the method of Scatchard (30). The unpaired Student's *t*-test was used for statistical analysis of the data.

Results

No significant or consistent differences were found during the perfusion or isolation of hepatocytes from male or female rat liver. Cell viability varied between 90% and 98% and was not sex related. Attachment of cells in the presence of 5% FCS for 3 h was also similar, ~75%. Observations with the phase contrast microscope indicated that the cells had flattened after 24 h in culture. Further spreading with the projection of numerous long processes had occurred after 48 h in culture. Flattening and spreading were more pronounced in the presence of EGF but occurred to the same extent in male and female hepatocytes. Protein and DNA content were measured in cultures maintained in MEM at 48 h after seeding. There was no significant difference between cultures of male and female hepatocytes. Protein content was 0.11 ± 0.03 mg/μg DNA for male cells and 0.11 ± 0.01 mg/μg DNA for female hepatocytes. The DNA content was 3.0 ± 0.35 and 2.9 ± 0.38 μg per dish for male and female hepatocytes, respectively. Furthermore, cell cultures derived from both male and female animals consisted almost exclusively of hepatocytes, as 98% of the cells stained for glucose-6-phosphatase (31) after 72 h in culture. Deoxyribonucleic acid synthesis of hepatocytes in monolayer cultures isolated from female rats, however, was much less than 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. The addition of EGF in the culture medium stimulated the [^{3}H]thymidine incorporation ninefold in male hepatocytes and two- to threefold in female hepatocytes compared to the incorporation observed when the hormone was not present in the culture medium. The addition of insulin to the EGF-supplemented medium did not change this picture drastically.

Slightly different results were obtained when DNA synthesis was compared in vitro in hepatocytes

Table 1. Incorporation of [^{3}H]Thymidine Into Primary Cultures of Hepatocytes Isolated From Male and Female Rats

Medium additions	[^{3}H]Thymidine incorporation (cpm/μg DNA) Male 48 h	Male 72 h	Female 48 h	Female 72 h
0	948 ± 123	1,630 ± 210	817 ± 253	1,593 ± 116
Insulin	931 ± 326	1,555 ± 96	940 ± 184	1,673 ± 231
EGF	5,210 ± 352[a]	12,556 ± 940[a]	1,486 ± 252[a]	5,617 ± 458[a]
Insulin ± EGF	8,435 ± 388[a,b]	19,231 ± 1,270[a,b]	2,078 ± 213[a,b]	6,195 ± 762[a]

DNA, deoxyribonucleic acid; EGF, epidermal growth factor. Hepatocyte isolation has been described under Materials and Methods. Cells were plated in MEM supplemented with 5% fetal calf serum and insulin. After 3 h the medium and floating cells were removed and serum-free MEM was added, plus the hormones as indicated. Cultures were exposed to 5 μCi [^{3}H]thymidine per milliliter for the last 24 h and processed after 48 or 72 h in culture for determination of deoxyribonucleic acid synthesis. The hormone concentrations were 10^{-7} M insulin and 10 ng/ml epidermal growth factor. For each medium triplicate culture dishes were set up. The numbers are the averages from nine dishes and 3 different animals ± SD. [a] Significantly different from base values ($p < 0.005$). [b] Significantly different from epidermal growth factor alone ($p < 0.01$).

Table 2. Incorporation of [³H]Thymidine Into Primary Cultures of Hepatocytes Isolated From Male and Female Rats 24 Hours After 70 Percent Hepatectomy

Medium additions	[³H]Thymidine incorporation (cpm/μg DNA)			
	Male		Female	
	48 h	72 h	48 h	72 h
0	3,204 ± 410	4,196 ± 478	1,007 ± 384	1,596 ± 326
Insulin	2,931 ± 316	5,047 ± 617	818 ± 184	1,586 ± 136
EGF	18,470 ± 1,920	27,150 ± 3,400	2,962 ± 363	11,419 ± 938
Insulin + EGF	25,416 ± 1,748[a,b]	34,889 ± 3,353[a,b]	4,248 ± 512[a,b]	13,996 ± 984[a,b]

DNA, deoxyribonucleic acid; EGF, epidermal growth factor. Culture conditions were the same as in Table 1. Hepatocytes were prepared 24 h after 70% of the liver had been removed as reported under Materials and Methods according to the method of Higgins and Anderson (Reference 22). For each medium, triplicate culture dishes were set up for each of the hepatocyte preparations. Cells were exposed to 5 μCi/ml [³H]thymidine for the last 24 h before the determination of deoxyribonucleic acid synthesis. The numbers for insulin and epidermal growth factor alone are the averages from six determinations from 2 different animals. The numbers with MEM alone and with insulin and epidermal growth factor are the averages from 12 determinations and 3 animals. All averages are shown ± SD. [a] Significantly different from base values ($p < 0.005$). [b] Significantly different from epidermal growth factor alone ($p < 0.01$).

isolated from male or female rats 24 h after partial hepatectomy (Table 2). In male hepatocytes from regenerating liver, regardless of incubation medium, DNA synthesis was increased two- to threefold over that observed in hepatocytes from normal liver. In females there was also a twofold 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 the EGF response of hepatocytes isolated from male or female regenerating liver was similar with respect to the "fold" increase in DNA synthesis, but was of a different magnitude.

Some of these experiments were recently repeated with 0.26 mM proline present in the medium. It has been reported that proline enhances DNA synthesis in hepatocytes maintained in primary culture (32,33). We found a 1.4- to 1.6-fold stimulation of [³H]thymidine incorporation due to the presence of proline. This degree of stimulation was the same whether hepatocytes were isolated from male or female rat liver.

To show that [³H]thymidine incorporation represents largely replicative DNA synthesis, autoradiography was done on several hepatocyte cultures. The results of this determination are shown in Figure 1 and it appears that the labeling index is proportional to [³H]thymidine incorporation. An example of labeled hepatocytes is shown in Figure 2. The labeling index is not affected by the several-fold differences in the thymidine pool, which would only affect the number of grains over each nucleus.

Further evidence for replicative DNA synthesis is provided by the increase of DNA content in the hepatocyte cultures grown in MEM supplemented with insulin and EGF as shown in Table 3. The DNA content remained rather constant over 72 h if cultures were maintained in MEM alone. After 48 and 72 h a significant increase in DNA content was observed in hepatocytes maintained in culture with insulin and EGF. Substantially less DNA content was found in female hepatocyte cultures, confirming the lower [³H]thymidine incorporation observed in the same culture conditions (Table 1).

Our findings that [³H]thymidine incorporation in the hepatocyte system was drastically inhibited by

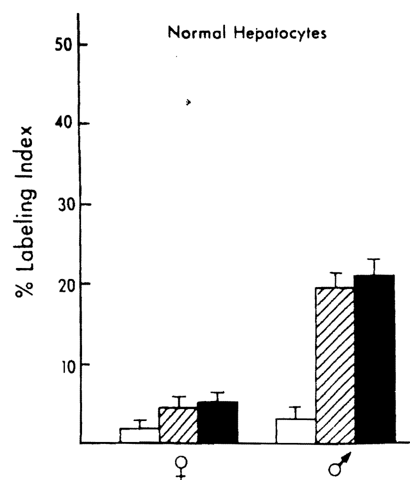

Figure 1. Labeling index of hepatocytes from male and female rats. Hepatocyte isolation has been described in Materials and Methods. Cells were plated in MEM supplemented with 5% FCS and insulin. After a 3-h attachment period, the medium was changed to serum-free MEM and hormones were added as indicated. Exposure to [³H]thymidine was from 48 to 72 h. Individual culture dishes were processed for autoradiography. In each dish a total of 1000 cells were counted to obtain the percentage of labeled cells. Each bar represents the average of 5 dishes ± SD. *Open bars*, MEM; *hatched bars*, MEM + EGF; *solid bars*, MEM + insulin + EGF.

FALK LIBRARY OF THE HEALTH PROFESSIONS

Figure 2. Examples of labeled hepatocytes exposed to [^{3}H]thymidine for 24 h from male rats grown in MEM supplemented with insulin and EGF for 72 h and stained for glucose-6-phosphatase. The nuclei are filled with black grains of [^{3}H]thymidine. The dark color of the cytoplasm indicated the positive stain. Phase contrast, ×200.

10 mM hydroxyurea is a further indication of replicative DNA synthesis in the hepatocytes. Contemporaneous addition of 10 mM hydroxyurea and [^{3}H]thymidine between 48 and 72 h in culture medium containing insulin and EGF inhibited DNA synthesis by 96% in male-derived hepatocytes and by 94% in female-derived hepatocytes. Exposure to hydroxyurea and [^{3}H]thymidine incorporation between 70 and 72 h resulted in 95% inhibition of DNA synthesis in hepatocytes derived from both sexes. Addition of hydroxyurea to cultures maintained in MEM alone inhibited DNA synthesis between 40% and 50%.

The possibility was considered that the lack of a 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 4. On the contrary, the numbers obtained with female rats were more consistent and at least of the same if not a higher magnitude. In addition, the labeling index determined at 28 h after partial hepatectomy was the same, with 33.2% and 34.4% labeled cells in male and female rats, respectively.

It was also considered that female hepatocytes in primary culture might respond better to higher or lower levels of EGF. A titration of EGF with both male and female hepatocytes is shown in Figure 3. For both preparations 10 ng/ml appears to be the concentration that maximally stimulates DNA synthesis. However, male hepatocytes respond to EGF to a significantly greater extent than do female hepatocytes.

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

Table 3. Increase in Deoxyribonucleic Acid Content in Male and Female Hepatocyte Cultures Grown in Different Media

Time in culture (h)	Male[a]		Female[a]	
	MEM	MEM + I + EGF	MEM	MEM + I + EGF
24	3.1 ± 0.22	3.2 ± 0.16	2.9 ± 0.21	2.8 ± 0.30
48	3.0 ± 0.37	4.23 ± 0.57[b]	2.9 ± 0.38	3.1 ± 0.25
72	3.1 ± 0.42	7.7 ± 0.37[b]	3.2 ± 0.45	4.9 ± 0.34[b]

EGF, epidermal growth factor. I, insulin. Culture conditions were as for Table 1. At indicated times, three dishes from each experiment were processed for deoxyribonucleic acid determination as described under Materials and Methods. The values are the average of seven determinations from two different experiments ± SD. [a] Values represent micrograms of deoxyribonucleic acid per culture dish. [b] Significantly different from base values (MEM) ($p < 0.005$).

Table 4. Deoxyribonucleic Acid Synthesis in Livers of Male and Female Rats at Different Times After Partial Hepatectomy

Time after 70% hepatectomy (h)	[^{3}H]Thymidine incorporation (cpm/μg DNA)	
	Male	Female
20	4,760 ± 670	9,890 ± 840
24	24,400 ± 5,720	27,520 ± 2,300
28	6,990 ± 1,250	5,850 ± 310

Seven-week-old rats weighing between 170 and 250 g had 70% of their livers removed between 7 and 9 AM. The animals were injected with 10 μCi [^{3}H]thymidine 1 h before the indicated times. The livers were removed and processed for deoxyribonucleic acid synthesis. The numbers shown for each time point are the averages from 6 different rats ± SD.

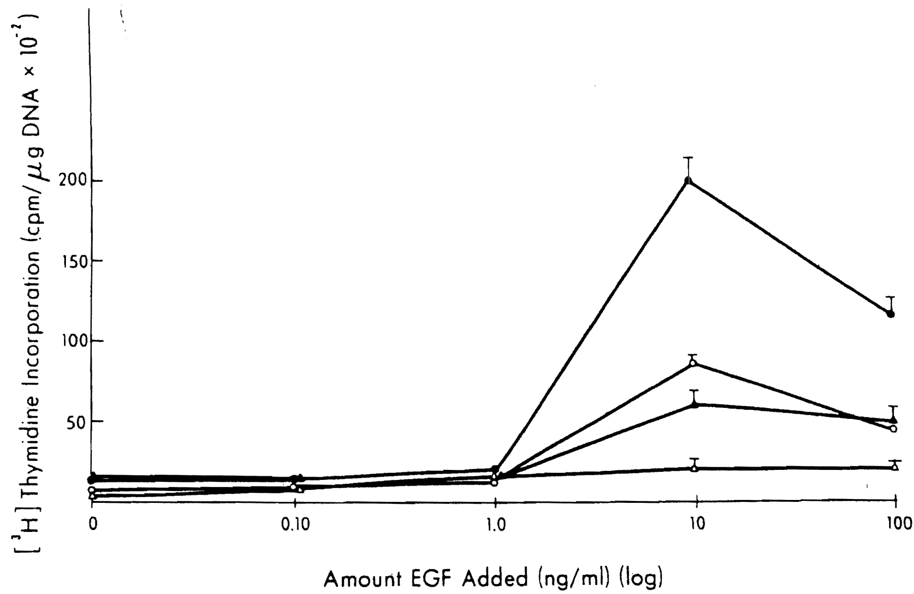

Figure 3. The effect of different EGF levels on DNA synthesis in hepatocytes from male or female rats. Conditions for the assay were the same as for Table 1. The medium was MEM with a constant level (10^{-7} M) of insulin. Exposure to [^{3}H]thymidine was from 24 to 48 h or from 48 to 72 h. Values shown were from six different determinations and two different hepatocyte preparations with the SD indicated by the t-bars. O-O, male hepatocytes at 48 h; ●-●, male hepatocytes at 72 h; △-△, female hepatocytes at 48 h; ▲-▲, female hepatocytes at 72 h.

The binding curve and Scatchard analysis are shown in Figure 4. Female hepatocytes bound more EGF than male hepatocytes: $B_{max} = 9.1 \times 10^{-10}$ M versus $B_{max} = 2.8 \times 10^{-10}$ M. Female hepatocytes, however, exhibited a significantly lower affinity for EGF ($K_d = 3.17 \times 10^{-8}$ M) than did male hepatocytes ($K_d = 5 \times 10^{-9}$ M). These B_{max} and K_d values were obtained from binding studies done on hepatocytes in culture for 24 h. Similar values were obtained when the binding studies were done using hepatocytes cultured in attachment medium for 3 h.

Epidermal growth factor binding studies were also done on male and female hepatocytes isolated 24 h after 70% hepatectomy. The Scatchard analysis is shown in Figure 5; in both cases only very low affinity binding is evident ($K_d = 1.37 \times 10^{-7}$ M for male hepatocytes, $K_d = 1.05 \times 10^{-7}$ M for female hepatocytes).

In the EGF binding studies on male hepatocytes, estrogen at physiologic concentrations of 10 pg/ml

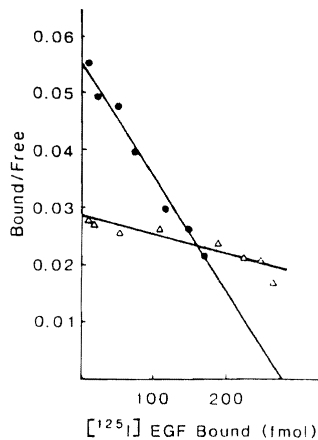

Figure 4. Epidermal growth factor binding. Scatchard analysis obtained from male and female hepatocytes. Culture and binding conditions are reported in Materials and Methods. ●-●, male hepatocytes; △-△, female hepatocytes.

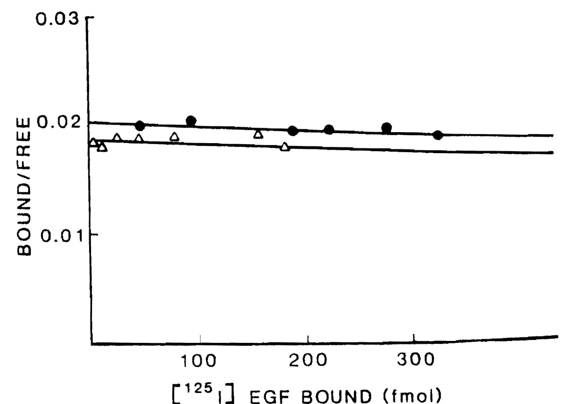

Figure 5. Epidermal growth factor binding. Scatchard analysis obtained from male, △-△, and female, ●-●, hepatocytes prepared 24 h after 70% hepatectomy.

FALK LIBRARY OF THE HEALTH SCIENCES

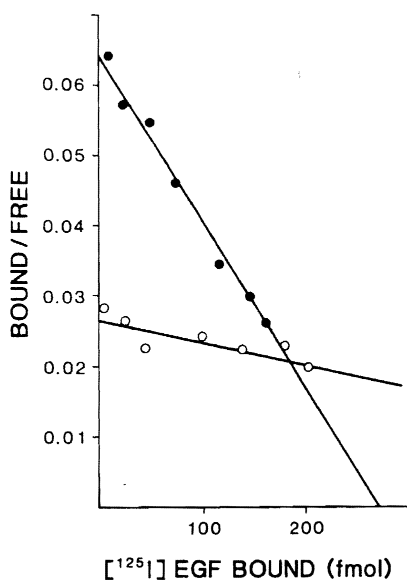

Figure 6. Epidermal growth factor binding. Scatchard analysis obtained from male hepatocytes incubated for 1 h without, ●-●, and with, ○-○, 10 pg/ml of estradiol.

and EGF were present simultaneously. The Scatchard analysis is shown in Figure 6. In the presence of estrogen, male hepatocytes show the same value of K_d (3.15×10^{-8} M) and B_{max} (8.55×10^{-10} M) observed in female hepatocytes, as is reported in Figure 4.

This result becomes even more significant in view of the finding that estrogen inhibits EGF-induced [^{3}H]thymidine incorporation in male hepatocytes as well (Table 5). Considerably higher levels of estrogen were needed to inhibit DNA synthesis than the levels required to inhibit EGF binding. One reason for this might be that [^{3}H]thymidine incorporation is measured over a 24-h period, present in culture between 24 and 48 h, with estrogen and EGF present from the time of plating. During the binding studies both estrogen and EGF are present for only 1 h. Another possible explanation is that the observed difference in EGF binding is only partially due to estrogen. It is unlikely that the inhibition of [^{3}H]thymidine incorporation by estrogen is a non-specific effect associated with the high concentration of steroids used. The additions of testosterone, up to 10 times the estrogen levels added, 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.

Discussion

Despite no apparent differences in the magnitude or onset of hepatic DNA synthesis in vivo in male and female rats after 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 morphologic appearance, DNA content, protein content, and [^{3}H]thymidine incorporation in the absence of EGF. On the other hand, female hepatocytes respond with increased DNA synthesis to the presence of EGF to a much lesser extent than male hepatocytes.

The results obtained with EGF binding studies provide a possible explanation for these findings. Binding studies performed using intact hepatocytes after 3 or 24 h 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 6, using male hepatocytes in the presence of estrogen at physiologic dosage, show EGF receptor behavior that is similar to that observed with female hepatocytes in the absence of hormones, and the data in Table 5 indicate that estrogen inhibits EGF-induced DNA synthesis in male hepatocytes. Male and female hepatocytes isolated 24 h after partial hepatectomy did not exhibit the difference in EGF binding. As shown in Figure 5, with both cell preparations there

Table 5. Inhibition of Epidermal Growth Factor-Induced Deoxyribonucleic Acid Synthesis by Estrogen in Primary Cultures of Hepatocytes Isolated From Male Rats

Estrogen addition (μg/ml)	[^{3}H]Thymidine incorporation (cpm/μg DNA)	Percentage of inhibition
0	8565 ± 350	—
5	3426 ± 102[a]	60
10	882 ± 65[a]	90
20	428 ± 35[a]	95

DNA; deoxyribonucleic acid; EGF, epidermal growth factor. Hepatocyte isolation has been described under Materials and Methods. Cells were plated in MEM supplemented with 5% fetal calf serum and insulin. After 3 h the medium and floating cells were removed and serum-free MEM supplemented with insulin (10^{-7} M) and epidermal growth factor (10 ng/ml) was added. Indicated amounts of estrogen were added at this time also and left in the medium until deoxyribonucleic acid synthesis determinations were done. Cultures were exposed to 5 μCi [^{3}H]thymidine per milliliter from 24 to 48 h and processed for deoxyribonucleic acid synthesis determination at 48 h. The numbers are the averages from nine dishes and 3 different animals ± SD. [a] Significantly different from control values ($p < 0.005$).

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 level of EGF receptor with high affinity. In fact, when EGF is added to hepatocytes that 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. Because EGF binding is different in male and female newly isolated hepatocytes and also in membrane preparations (34), factors other than EGF seem to be responsible for in vivo regeneration.

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

In a series of experiments dealing with rat liver regeneration, we have previously shown significant changes in testosterone and estrogen in the blood of male rats after 70% hepatectomy (35,36). These results were characterized by a decrease of serum testosterone and an increase of serum estrogen in the blood of the animals associated with a change of sex-related receptors of hepatocytes. Twenty-four hours after hepatectomy we found an almost complete disappearance of androgen receptors with a significant increase in total and nuclear-associated estrogen receptors. In addition, we report an almost complete disappearance in the liver of estrogen-2-hydroxolase and male-specific estrogen binding proteins that are known to promote a rapid binding as well as metabolism of estrogen. This clearly shows distinct feminization of the hepatectomized rat, and suggests a role for estrogens in the maintenance or control of hepatocyte regeneration.

The data reported here suggest that the decrease of EGF receptor binding that occurs in male rat liver membranes after hepatectomy (11,37) may be an expression of the increased estrogen level in the blood as well as the increase of nuclear estrogen receptor in hepatectomized liver that has lost the ability to metabolize the estrogen.

It appears that low EGF binding is important for endogenous hepatomitogens to be effective in inducing proliferation activity. This enables us to explain our results reported earlier (11) that hepatocytes isolated from regenerating liver have increased EGF-induced DNA synthesis at conditions of low EGF binding.

FALK LIBRARY

References

1. Richman RA, Claus TH, Pilkis SJ, Friedman DL. Hormonal stimulation of DNA synthesis in primary cultures of adult rat hepatocytes. Proc Natl Acad Sci USA 1976;73:3589–93.
2. Koch KS, Leffert HL. Increased sodum ion influx is necessary to initiate rat hepatocyte proliferation. Cell 1979;18:153–63.
3. Tomita V, Nakamura T, Ichihara A. Control of DNA synthesis and ornithine decarboxylase activity by hormones and amino acids in primary cultures of adult rat hepatocytes. Exp Cell Res 1981;135:363–71.
4. Michalopoulos G, Cianciulli HD, Novotny AR, Kligerman AD, Strom SC, Jirtle RL. Liver regeneration studies with rat hepatocytes in primary culture. Cancer Res 1982;42:4673–82.
5. Hasegawa K, Watanabe K, Koga M. Induction of mitosis in primary cultures of adult rat hepatocytes under serum-free conditions. Biochem Biophys Res Commun 1982;104:259–65.
6. McGowan JA, Bucher NRL. Pyruvate promotion of DNA synthesis in serum-free primary cultures of adult rat hepatocytes. In Vitro 1983;19:159–66.
7. Enat R, Jefferson DM, Ruiz-Opazo N, Gatmaitan Z, Leinwand LA, Reid LM. Hepatocyte proliferation in vitro: its dependence on the use of serum-free hormonally defined medium and substrate of extra-cellular matrix. Proc Natl Acad Sci USA 1984;81:1411–5.
8. McGowan JA, Strain AJ, Bucher NRL. DNA synthesis in primary cultures of adult rat hepatocytes in a defined medium: effects of epidermal growth factor, insulin, glucagon and cyclic AMP. J Cell Physiol 1981;103:353–63.
9. Koch KS, Shapiro P, Skelly H, Leffert HL. Rat hepatocyte proliferation is stimulated by insulin-like peptides in defined medium. Biochem Biophys Res Commun 1982;109:1054–60.
10. McGowan JA, Russell WE, Bucher NRL. Hepatocyte DNA replication: effect of nutrients and intermediary metabolites. Fed Proc 1984;43:131–3.
11. Francavilla A, Ove P, Polimeno L, Sciascia C, Coetzee ML, Starzl TE. Epidermal growth factor and proliferation in hepatocytes in primary cultures isolated at different times after partial hepatectomy. Cancer Res 1986;46:1318–23.
12. Rozengurt E, Brown KD, Pettican P. Vasopressin inhibition of epidermal growth factor binding to cultured mouse cells. J Biol Chem 1981;256:716–22.
13. Wharton W, Leof E, Pledger WJ, O'Keefe EJ. Modulation of the epidermal growth factor receptor by platelet-derived growth factor and choleragen: effects on mitogenesis. Proc Natl Acad Sci USA 1982;79:5567–71.
14. Brown KD, Blay J, Irvine RF, Heslop JP, Berridge MJ. Reduction of epidermal growth factor receptor affinity by heterologous ligands: evidence for a mechanism involving the breakdowns of phosphoinositides and the activation of protein kinase C. Biochem Biophys Res Commun 1984;123:377–84.
15. Lin Q, Blaisdell Y, O'Keefe F, Earp HS. Insulin inhibits the glucocorticoid-mediated increase in hepatocyte EGF binding. J Cell Physiol 1985;119:267–72.
16. Wolffe AP, Rakhmet IB, Jamshed RT. Inhibition by estradiol of binding and mitogenic effect of epidermal growth factor in primary cultures of *Xenopus* hepatocytes. Mol Cell Endocrinol 1985;40:167–73.
17. Bone SN III, Michalopoulos G, Jirtle RL. Ability of partial hepatectomy to induce γ-glutamyltranspeptidase in regenerated and transplanted hepatocytes of Fischer 344 and Wistar–Furth rats. Cancer Res 1985;45:1922–8.
18. Short JA, Brown RF, Husakova A, Gilbertson J, Zemel R, Lieberman I. Induction of deoxyribonucleic acid synthesis in the liver of the intact animal. J Biol Chem 1972;1757–66.
19. Norstedt G, Eneroth P, Gustafsson J, Hokfelt T, Skett P.

Hypothalamo-pituitary regulation of hepatic prolactin receptors in the rat. Brain Res 1980;192:77–88.

20. Eacho PI, Weiner M. Metabolism of p-nitroanisole and aniline in isolated hepatocytes of stretptozotocin-diabetic rats. Drug Metab Dispos 1980;8:385–9.
21. Eagon PK, Porter LE, Francavilla A, DiLeo A, Van Thiel DH. Estrogen and androgen receptors in liver; their role in liver disease and regeneration. Sem Liver Dis 1985;5:59–69.
22. Higgins GM, Anderson RM. Restoration of the liver of the white rat following partial surgical removal. Arch Pathol 1931;12:186–202.
23. Seglen PO. Preparation of isolated rat liver cells. Methods Cell Biol 1976;13:29–83.
24. Jirtle L, Michalopoulos G, McLain JR, Crowley J. Transplantation of parenchymal hepatocytes exposed to ionizing radiation. Cancer Res 1981;41:3512–8.
25. Setaro F, Morley CGD. A modified fluorometric method for the determination of microgram quantities of DNA from cell or tissue cultures. Anal Biochem 1976;71:313–7.
26. Michalopoulos G, Houck KA, Dolan ML, Luetteke NC. Control of hepatocyte replication by two serum factors. Cancer Res 1984;44:4414–9.
27. Coetzee ML, Short J, Klein K, Ove P. Correlation of circulating levels of a serum protein with triiodothyronine levels and hepatoma growth. Cancer Res 1982;42:155–60.
28. Haigler HJT, Maxfield FR, Willingham MC, Pastan I. Dansylcadaverine inhibits internalization of [^{125}I] epidermal growth factor in Balb 3T3 cells. J Biol Chem 1980;225:1239–41.
29. Lowry OH, Rosebrough NY, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951;193:265–75.
30. Scatchard G. The attractions of proteins for small molecules and ions. Ann NY Acad Sci 1949;151:660–72.
31. Wachstein M, Meisel E. Histochemistry of hepatic phosphatase at physiologic pH. Am J Clin Pathol 1957;27:13–23.
32. Nakamura T, Teramoto H, Tomita Y, Ichihara A. L-Proline is an essential amino acid for hepatocyte growth in cultures. Biochem Biophys Res Commun 1984;122:884–91.
33. Houck KA, Michalopoulos G. Proline is required for the stimulation of DNA synthesis in hepatocyte cultures by EGF. In Vitro Cell Dev Biol 1985;21:121–4.
34. Benveniste R, Carson SA. Binding characteristics of epidermal growth factor receptors in male and female rat liver cell membrane preparations. Mol Cell Endocrinol 1985;41:147–51.
35. Francavilla A, Dileo A, Eagon PK, et al. Regenerating rat liver: correlations between estrogen receptor localization and deoxyribonucleic acid synthesis. Gastroenterology 1984;86:552–7.
36. Francavilla A, Eagon PK, DiLeo A, et al. Sex hormone related functions in regenerating male rat liver. Gastroenterology 1986;91:1263–70.
37. Earp HS, O'Keefe EJ. Epidermal growth factor receptor number decreases during rat liver regeneration. J Clin Invest 1981;67:1580–3.