0270-9139/89/0904-0614$02.00/0
HEPATOLOGY

1004

Vol. 9, No. 4, pp. 614–620, 1989
Printed in U.S.A.

The Effect of Estrogen and Tamoxifen on Hepatocyte Proliferation *in Vivo* and *in Vitro*

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We have previously shown that changes in estrogen-hepatocyte interaction occur during liver regeneration. Following 70% hepatectomy, estrogen levels in the blood were elevated, the number of estrogen receptors in the liver was increased and there was an active translocation of estrogen receptors from the cytosol to the nucleus. The injection of tamoxifen, an estrogen antagonist, inhibits hepatocyte proliferation following partial hepatectomy. The administration of 1 μg tamoxifen per gm body weight at zero time or 6 hr after the operation resulted in a significant inhibition both of DNA synthesis and of the number of cells in mitosis. Injections of tamoxifen 12 hr or later after the operation had no effect. Concomitant injections of equimolar amounts of estrogen abolished the inhibition by tamoxifen. The effects of estrogen and tamoxifen were also tested on hepatocytes in primary culture. Estrogens in the presence of 5% normal rat serum stimulated hepatocyte DNA synthesis as determined by [^{3}H]thymidine incorporation and the labeling index, whereas epidermal growth factor-induced DNA synthesis in the absence of normal rat serum was strongly inhibited. Tamoxifen, in contrast, inhibited DNA synthesis of hepatocytes in the presence of 5% normal rat serum and reversed the stimulatory effect of estrogen in the same system. Attempts to elucidate the mechanism of tamoxifen inhibition *in vitro* indicated that one effect of tamoxifen is to prevent the amiloride-sensitive Na^+ influx necessary to initiate hepatocyte proliferation.

There is now considerable experimental evidence that sex hormones may modulate the process of liver regeneration. We have demonstrated an increase in estrogen receptors in the rat liver after 70% partial hepatectomy (1, 2). We have extended these results to demonstrate a complete interdependence of sex hormones and their receptors in the liver during the regenerative process (3). Testosterone levels in the serum decrease precipitously following 70% hepatectomy, whereas concomitant serum estrogen levels become significantly elevated. Partial hepatectomy induces significant alterations in the absolute number of hepatic sex hormone receptors. There was a significant and rapid reduction of cytosolic and nuclear androgen receptors, whereas estrogen receptors increased rapidly in the nuclear fraction (3). The degree of alteration in estrogenic physiology after hepatectomy is further emphasized by the reduction of the cytosolic male estrogen-binding protein and the microsomal enzyme estrogen 2-hydroxylase, which represent two systems responsive to androgenic modulation. Despite these consistent changes, the exact role of estrogens and significance in the overall process of liver regeneration remain to be elucidated. The experiments reported here were designed to define further the role of estrogenic hormones and their receptors in liver regeneration by assessing the effect of tamoxifen, an estrogen antagonist, in both *in vivo* and *in vitro* models.

MATERIALS AND METHODS

Animals. Male Fischer F344 rats weighing between 180 and 200 gm were obtained from Hilltop Lab Animals, Inc. (Scottdale, PA). The animals were housed in a temperature- and light- (6 a.m. to 6 p.m.) controlled room and received food and water *ad libitum*. Seventy per cent partial hepatectomy (PH) was performed as previously described by Higgins and Anderson (4). Sham hepatectomy consisted of laparotomy and gentle manipulation of the liver. All surgical procedures were performed between 8:00 and 10:00 a.m. All injections were administered by the intraperitoneal route.

Materials. Collagenase (Type I) (140 to 170 units per mg) was purchased from Worthington Diagnostic Systems (Freehold, NJ). Eagle's MEM and fetal calf serum (FCS) were purchased from GIBCO Laboratories (Grand Island, NY). Insulin, HEPES, pyruvic acid, tamoxifen and estrogen (17β-estradiol) were purchased from Sigma Chemical Co. (St. Louis, MO). Epidermal growth factor (EGF) was purchased from Collaborative Research, Inc. (Bedford, MA). [*methyl*-^{3}H]Thymidine (50 to 80 Ci per mmole), [$^{22}Na^+$]sodium chloride in water (532 mCi per mg) and ^{125}I-EGF (150 to 200 μCi per μg) were purchased from New England Nuclear (Boston, MA). Gentamicin was purchased from Elkins-Sinn, Inc. (Cherry Hill, NJ). Aqueous counting solution was purchased from Amersham (Arlington Heights, IL).

Received August 12, 1987; accepted October 10, 1988.
Supported by awards from the Veterans Administration, Grant AM-29961 from the National Institutes of Health and Grant 87/01291-44 from Consiglio Nazionale delle Ricerche, Italy.
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Drug Administration. Both tamoxifen and estradiol were dissolved in 100% ethanol at a concentration of 10 mg per ml. For *in vivo* experiments, a solution of 1:25 (v/v) in saline was prepared from the above concentration immediately before the i.p. injection. Control animals received an injection of a saline solution containing 4% ethanol.

For *in vitro* experiments, 10 mg estradiol or tamoxifen were dissolved in 1 ml ethanol and were diluted in culture medium (0.4 to 1:1,000 v/v). It was essential to keep the amount of ethanol as small as possible, as even 0.5% of ethanol was quite toxic for the cells. In all experiments in which tamoxifen or estrogen was added, control culture received the same amount of ethanol used for the addition of tamoxifen and estrogen. [^{3}H] Thymidine incorporation was the same in the absence or presence of these levels of ethanol.

This study has been approved by the Research and Development Committee of our institution.

In Vivo [^{3}H]Thymidine Incorporation. To measure hepatic DNA synthesis, rats were given intravenous injections of 50 μCi [^{3}H]thymidine per 200 gm body weight 1 hr prior to killing; 1 hr later, the animals were killed and the incorporation of [^{3}H]thymidine and the percentage of labeled nuclei were determined as previously described (5, 6).

Binding Studies. Purified plasma membrane fractions were prepared by the method of Neville (7) from normal rats and hepatectomized rats treated with or without tamoxifen. The purity of the membrane preparation was assessed by the assay of 5′-nucleotidase activity. Radiolabeled ligands were incubated with 9 to 25 μg membrane protein for 40 min at 24°C in 0.2 ml Dulbecco's phosphate-buffered saline containing 0.1% bovine serum albumin. Duplicate samples were collected on Millipore EGWP filters (Millipore Corp., Bedford, MA). Each determination was corrected for nonspecific binding by subtraction of ^{125}I-EGF bound in the presence of 1 μM native EGF added 10 min prior to the labeled material (8). The B_{max} and K_d were calculated by Scatchard analysis (9).

Hepatocytes in Primary Culture. Hepatocytes were isolated from 7-week-old male rats weighing between 180 and 200 gm, by a modification of the *in situ* two-step collagenase perfusion technique of Seglen (10) and Jirtle et al. (11) and modified further, as previously described (12). The hepatocytes were dispersed and washed twice with cold Ca^{++}-free perfusion buffer and resuspended in basal medium (MEM) supplemented with pyruvate (1 mM), aspartate (0.2 mM), serine (0.2 mM), gentamicin (40 mM), proline (0.26 mM), insulin (10^{-7} M) and 5% FCS. Viability was determined by trypan blue exclusion (12), and only preparations having greater than 90% viability were used. Cell number was determined with a hemocytometer.

The cells were plated at a cell density of 6.5×10^4 per well in Falcon "Primaria" 24-well tissue culture plates in 0.5 ml medium and maintained at 37°C in a 5% CO_2 atmosphere. The surface area of each well was 2 cm^2. Following a 3-hr attachment period, the medium was aspirated, 0.5 ml serum-free basal medium was added and hormonal additions were made as indicated. EGF and insulin were at a concentration of 10 ng per ml and 10^{-7} M, respectively. In the experiments with tamoxifen, the culture medium was always supplemented with 5% normal male rat serum (NRS). This concentration of serum was necessary to protect hepatocytes from the cytotoxic effects of tamoxifen observed in serum-free medium.

In Vitro [^{3}H]Thymidine Incorporation. To determine *in vitro* DNA synthesis, 3 μCi [^{3}H]thymidine were added to each well and maintained from 24 to 48 hr or from 48 to 72 hr of the culture period. Each experimental group consisted of six wells. When the cells were harvested, three wells were used to determine DNA content by the microfluorometric method of Setaro and Morley (13), and the three remaining wells of each group were treated as described by Michalopoulos et al. (14) to measure the DNA synthesis (incorporation of [^{3}H]thymidine).

Autoradiography. For autoradiographic studies, hepatocytes were plated in 35-mm dishes as previously described (12, 15). [^{3}H]Thymidine, 7.5 μCi per dish, was present for 24 hr prior to fixation. Fixed cells in the dishes were covered with emulsion (Kodak NTB3), and the dishes were developed after 10 days. The labeling index was determined by counting a total of 1,000 cells per dish.

Measurement of $^{22}Na^+$ Uptake. Uptake of $^{22}Na^+$ was determined essentially as described by Koch and Leffert (16) using hepatocytes cultured in 35-mm Primaria culture dishes. Following a 3-hr attachment period, the medium was removed and 1.5 ml per well of serum-free basal MEM were added. The plates were left at 37°C in a 5% CO_2 atmosphere for 20 hr. The plates were then washed twice with serum-free MEM containing 0.4 mM ouabain, and/or 0.4 mM amiloride where indicated. The basic incubation medium was MEM plus 10^{-7} M insulin, 0.4 mM ouabain and 2 μCi per ml $^{22}Na^+$. Where indicated, estrogen and 5% NRS were added as the mitogens and tamoxifen as potential inhibitor. Duplicate dishes were set up in each experiment in the presence of 0.4 mM amiloride. Incubation was for 90 min at 37°C in a 5% CO_2 atmosphere. Following the 90-min incubation, the cultures were washed with 1 ml of cold 250 mM sucrose, pH 6.0, and the radioactivity was extracted by the addition of 1 ml of 330 mM NaOH at 37°C for 30 min. The supernatant was transferred to scintillation vials containing 0.1 ml 40% trichloroacetic acid and 10 ml aqueous scintillation solution, and radioactivity was determined in a Packard scintillation counter.

Other Methods. Proteins were determined by the method of Lowry et al. (17). The unpaired Student's t test was used for statistical analysis of the data.

RESULTS

Tamoxifen administration resulted in an inhibition (77%) of the peak incorporation of [^{3}H]thymidine and of DNA synthesis normally observed in rats at 20 to 24 hr following PH (Table 1). DNA synthesis in the livers of sham-operated rats was not affected by tamoxifen. This latter result was to be expected, since the DNA synthesis in the livers of normal or sham-operated rats is very low; thus, an inhibition would not be obvious.

Administration of tamoxifen immediately after PH was not critical for the inhibition of DNA synthesis. As

TABLE 1. Effect of tamoxifen on liver regeneration

Treatment	No. of rats	[^{3}H]Thymidine incorporation (cpm/μg DNA)
Sham hepatectomy	7	7,500 ± 1,260
Sham hepatectomy + tamoxifen	6	6,950 ± 1,320
70% hepatectomy (control)	10	82,270 ± 25,820
70% hepatectomy + tamoxifen	10	19,195 ± 12,650[a]

A 70% hepatectomy or a sham hepatectomy was performed on male rats between 8 and 10 a.m. One microgram of tamoxifen per gm body weight of rat was injected i.p. immediately after the operation. Rats were given i.p. infections of 50 μCi [^{3}H]thymidine at 23 hr after the operations and killed 1 hr later. The numbers are the averages ± S.D.

[a] Significantly different from the 70% hepatectomy control value ($p < 0.01$).

can be seen in Figure 1, injection of tamoxifen at 6 hr after PH still resulted in a significant inhibition of [^{3}H] thymidine incorporation. Tamoxifen administration at 12 or 18 hr following partial hepatectomy, however, was no longer inhibitory. Also shown in Figure 1 are autoradiographic data confirming the [^{3}H]thimidine incorporation results. These findings suggest that an early event in the hepatic regenerative process may be sensitive to tamoxifen.

The inhibitory effect of tamoxifen could be reversed by the simultaneous administration of an equimolar amount of estradiol as shown in Figure 2. In the presence of tamoxifen, hepatic DNA synthesis was only 23% of that of the control rats which had not received any drug injections. The simultaneous injection of estradiol with tamoxifen reversed the inhibitory effect of tamoxifen and restored normal DNA synthesis as induced by partial hepatectomy.

Our finding that estradiol prevents the tamoxifen inhibition of DNA synthesis is further supported by the results shown in Figure 3. In male rats, the inhibition of DNA synthesis by tamoxifen lasts until 32 hr after partial hepatectomy, whereas in female rats inhibition is apparent only at 24 hr after the operation. Thus, the tamoxifen inhibition is of limited duration. These data

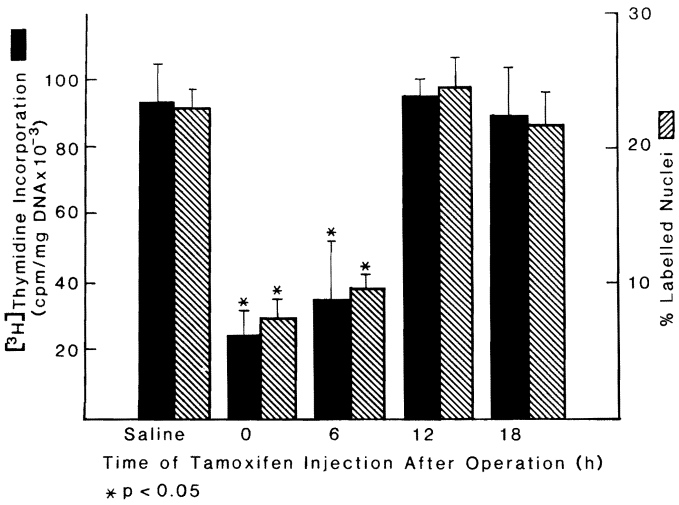

FIG. 1. Administration of tamoxifen at different times after 70% partial hepatectomy. All operations were performed at time 0, between 8 and 10 a.m. Injections of 1 μg per gm body weight tamoxifen were administered at indicated times after operation. All animals were given injections of 50 μCi [^{3}H]thymidine at 23 hr after the operation. One hour later, animals were killed, and hepatic DNA synthesis and labeled nuclei were determined. The values represented by the *bar* are means of six rats ± S.D. * = significantly different from control value, $p < 0.05$.

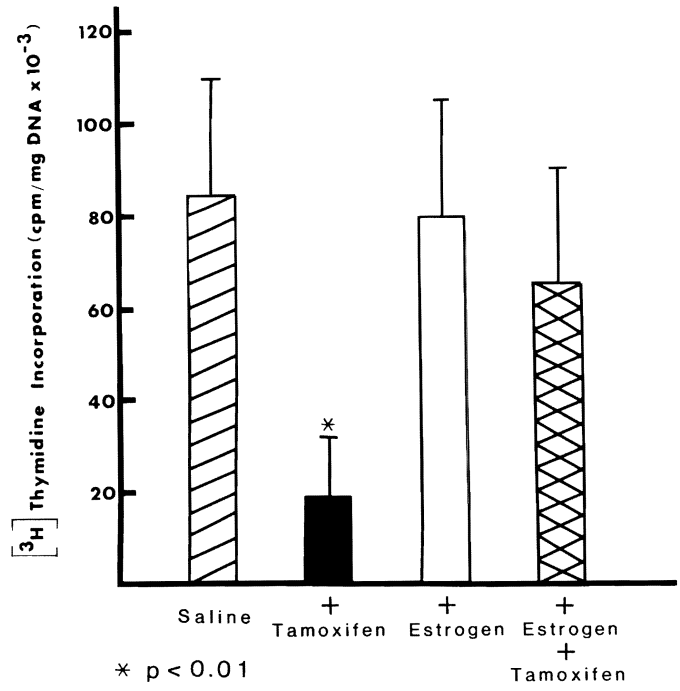

FIG. 2. Reversal of tamoxifen inhibition of DNA synthesis by estrogen. All animals had 70% of the liver removed between 8 and 10 a.m. Where indicated, tamoxifen (1 μg per gm body weight) and/or estradiol (0.8 μg per gm body weight) were injected at zero time after the surgical procedure. DNA synthesis at 24 hr was determined as indicated for Fig. 1. The values represented by the *bars* are the means from at least five rats ± S.D. * = significantly different from control value, $p < 0.01$.

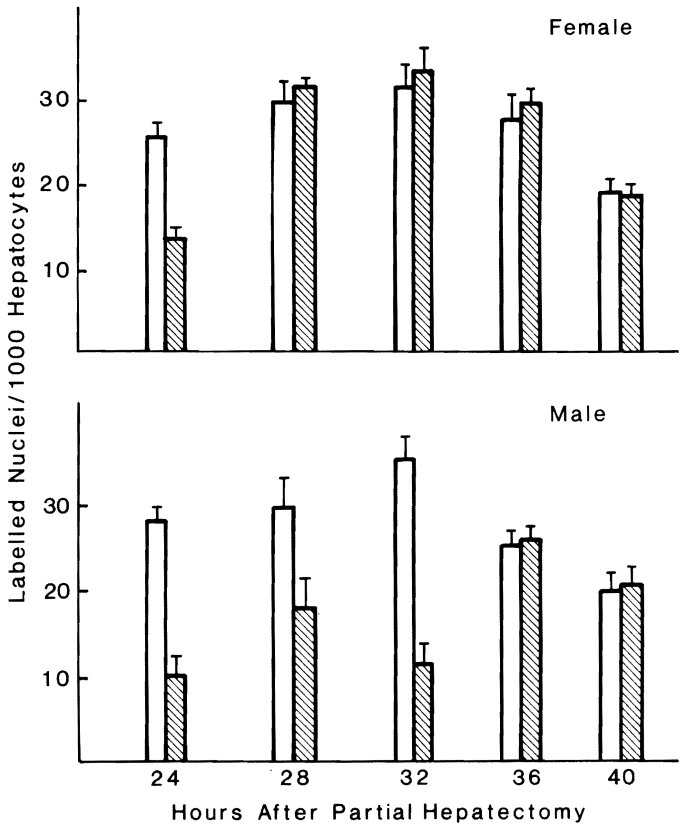

FIG. 3. Duration of tamoxifen inhibition of hepatocyte proliferation activity *in vivo* expressed as labeled nuclei following partial hepatectomy in male and female rats. All operations were performed between 8 and 10 a.m. Tamoxifen (1 μg per gm body weight) was injected at zero time after the operation. All animals were given injections of 50 μCi [^{3}H]thymidine 1 hr before they were killed. At specified times after partial hepatectomy, indicated on the *abscissa*, the animals were killed and labeled nuclei were determined as described (6). The values represented by each *bar* were derived from four controls and four tamoxifen-injected rats. *Open bars* = controls; *hatched bars* = tamoxifen-injected animals.

also indicate that tamoxifen does not kill the hepatocytes. Liver weight was almost fully restored at 10 days after partial hepatectomy, whether tamoxifen had been injected at the time of the operation or not.

As demonstrated in Table 2, tamoxifen also inhibited DNA synthesis drastically and in a dose-dependent fashion when added to hepatocyte cultures in the presence of 5% NRS. The results in Figure 4 demonstrate that the addition of tamoxifen as late as 36 hr after plating caused a 40% inhibition. Tamoxifen did not appear to be cytotoxic in our experimental conditions as evaluated by the trypan blue exclusion test and DNA amount per dish (data not shown).

Table 3 reports the effect of estrogens on hepatocyte proliferation in the presence of EGF in serum-free medium. At the concentrations shown, estrogen inhibited EGF-induced DNA synthesis, as previously demonstrated (18). In contrast, increased DNA synthesis and an increase in the labeling index was observed when estrogen was administered in the presence of 5% NRS. This increase in DNA synthesis due to estrogen can be inhibited by tamoxifen, as shown in Table 4. DNA synthesis stimulated by estrogens in the presence of 5% NRS was completely inhibited by 4 μM tamoxifen.

To understand the possible mechanism of tamoxifen effect, we investigated whether this drug would alter EGF receptor affinity or distribution. We compared EGF binding on isolated plasma membranes of normal rat liver with that of regenerating rat liver, injected with saline or tamoxifen. Our results indicated that tamoxifen had no effect on the down-regulation of EGF receptors following partial hepatectomy (8, 18). For normal rats, we found EGF binding values of $B_{max} = 1.2 \pm 0.3$ pmoles per mg protein and $K_d = 1.08 \pm 0.12$ nM. For regenerating rat liver, we found $B_{max} = 0.56 \pm 0.12$ pmole per mg protein and $K_d = 1.32 \pm 0.13$ nM, not significantly different from those observed in hepatectomized rats treated with tamoxifen ($B_{max} = 0.48 \pm 0.15$ pmoles per mg; $K_d = 1.28 \pm 0.13$ nM).

TABLE 2. Inhibition of DNA synthesis in hepatocytes in primary culture in the presence of 5% normal rat serum

Amount of tamoxifen added (μM)	[^{3}H]Thymidine incorporation (cpm/μg DNA)	% labeled nuclei
0	54,730 ± 5,460	8 ± 2
2.5	43,218 ± 3,910	6 ± 1.5
5	21,007 ± 3,008[a]	4 ± 0.2[a]
10	13,338 ± 874[a]	2.5 ± 0.25[a]

The preparation of hepatocyte cultures and the assay conditions are described in "Materials and Methods." The numbers are means of at least four different determinations ± S.D. Wells are exposed to 3 μCi [^{3}H]thymidine from 24 to 48 hr in culture.

[a] Significantly different from the value obtained using no tamoxifen ($p < 0.01$).

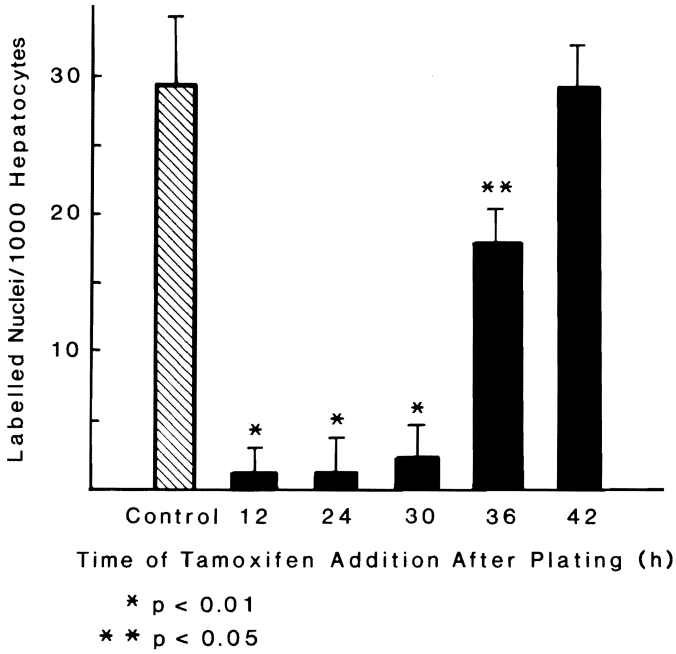

FIG. 4. Effect of tamoxifen added to hepatocytes at various times after plating. Hepatocytes were isolated and plated as described in "Materials and Methods." After 3 hr, the attachment medium was replaced with NRS-containing basal medium. At indicated times, three wells for each point received tamoxifen (4 mM). All wells received 3 μCi [^{3}H]thymidine at 24 hr. The cells were harvested at 48 hr. The values shown are the averages of at least three determinations with individual values within 5% of the mean shown. Significantly different from control value: * = $p < 0.01$; ** = < 0.05.

TABLE 3. Effect of estrogen on hepatic proliferation in the presence or absence of EGF and 5% normal rat serum

Amount 17β-estradiol added (μM)	[^{3}H]Thymidine incorporation (cpm/μg DNA)		% labeled nuclei	
	+ EGF	+ 5% NRS	+ EGF	+ 5% NRS
None	67,245 ± 5,350	60,321 ± 7,750	10 ± 4	9 ± 3
2.5	51,119 ± 6,350	90,175 ± 7,350	9.1 ± 2.7	11 ± 2
5	36,471 ± 1,850[a]	119,803 ± 11,320[a]	6.8 ± 2	14 ± 3
10	20,984 ± 1,820[a]	132,616 ± 9,180[a]	3.1 ± 1[a]	16 ± 2[a]

The assay conditions were as for Table 2. After a 3-hr attachment period, the medium was removed and replaced with MEM plus insulin containing either EGF (10 ng per ml) or 5% NRS. The values are the means from three determinations ± S.D.

[a] Significantly different from the control value (none) ($p < 0.005$).

TABLE 4. Effect of tamoxifen on estrogen-stimulated DNA synthesis in hepatocytes in primary culture in the presence of normal rat serum

Additions	Amount (μM)	[^{3}H]Thymidine incorporation (cpm/μg DNA)
None		41,000 ± 4,100
17β-Estradiol	2.5	48,000 ± 3,250
17β-Estradiol	5	58,120 ± 3,120
17β-Estradiol	10	72,100 ± 8,200
Tx[a]	5	13,000 ± 950
Tx (10 μM) + 17β-estradiol	2.5	5,600 ± 1,510
Tx (10 μM) + 17β-estradiol	5	9,100 ± 910
Tx (10 μM) + 17β-estradiol	10	13,120 ± 1,350

Assay conditions were as for Table 2. After a three-hr attachment period, the medium was removed and replaced with MEM + insulin containing 5% normal rat serum. The values are the means of three determinations ± S.D.

[a] TX = tamoxifen.

TABLE 5. Inhibition of $^{22}Na^+$ uptake by tamoxifen in hepatocytes maintained in primary culture

Incubation medium	$^{22}Na^+$ uptake (cpm/5 × 10^5 cells)	
	(−) Amiloride	(+) Amiloride (0.4 m*M*)
MEM	2,542 ± 250	2,858 ± 420
MEM + 5% NRS	2,866 ± 186	2,010 ± 120
MEM + estrogen (10 μM)	3,072 ± 180	2,675 ± 190
MEM + estrogen (10 μM) + 5% NRS	3,820 ± 150	2,820 ± 205
MEM + 5% NRS + Tx (10 μM)	1,212 ± 96[a]	1,157 ± 120[a]
MEM + estrogen (10 μM) + 5% NRS + Tx (10 μM)	1,025 ± 83[b]	950 ± 141[b]

The isolation and culture conditions of hepatocytes and the determination of $^{22}Na^+$ uptake have been described in "Materials and Methods." Following the 3-hr attachment period, the medium was removed and the cells were incubated in MEM for 20 hr. The indicated additions were then made, including 2 μCi $^{22}Na^+$ per dish, 0.4 m*M* ouabain and insulin, and the cells were incubated for 90 min. The numbers are the means from six dishes ± S.D. Tx = tamoxifen.

[a] Significantly different from the value obtained using MEM + 5% NRS ($p < 0.05$).

[b] Significantly different from the value obtained using MEM + estrogen (10 μM) + 5% NRS.

The experiment reported in Table 5 shows the effect of tamoxifen on amiloride-sensitive Na^+ flux, which is known to be one of the earliest phenomena occurring in hepatocyte proliferation (16). It has been well established that there is an increase Na^+ flux into hepatocytes due to the presence of mitogens in culture media and that this increase can be abolished by 0.4 m*M* amiloride. Our results indicate that Na^+ amiloride-sensitive flux, evident in the presence of the mitogens 5% NRS or estrogen (10 μM), is inhibited by tamoxifen.

DISCUSSION

Since Higgins and Anderson (4) originally reported the technique of partial hepatectomy in rats, investigators have been studying the mechanisms that regulate hepatic regeneration. During the last 10 years, hormones have assumed a prominent role as factors related in some way to hepatic regeneration after partial hepatectomy. It has been suggested that hormonal agents such as insulin, glucagon and epidermal growth factor may initiate the process of regeneration (19–28); however, hormones such as parathyroid hormone (27), calcitonin (23), iodothyronines (28) and glucocorticoids (29) also have been shown to influence the hepatic regenerative response.

In a recent series of studies, our group reported several important relationships between steroid receptors and liver function (30–32), and particularly the changes in sex hormones and their receptors during liver regeneration (1–3). First, we described changes in cytosolic and nuclear estrogen receptors in regenerating male rat liver during the first 72 hr after 70% partial hepatectomy (1). These data demonstrated that a significant increase in the number and nuclear localization of hepatic estrogen receptors was concurrent with the proliferative activity of the regenerating liver.

More recently, significant variations in serum androgen levels and changes in the hepatic distribution of the specific cytosolic and nuclear androgen receptors have been reported during liver regeneration in male rats (3). Concomitantly, testosterone levels decreased precipitously following 70% hepatectomy. As a further demonstration of the process of feminization in regenerating rat liver, we have also described a reduction in the cytosolic male estrogen-binding protein and of the microsomal enzyme, estrogen 2-hydroxylase, two systems responsive to androgenic modulation.

Our most recent results also determined that estrogen decreased the number of EGF receptors of male hepatocytes in culture. We have shown that this effect accounts for the inability of EGF to stimulate hepatocyte proliferation *in vitro* in the presence of estrogen and also helps explain the difference in liver EGF receptors, found by us and others, between male and female hepatocytes (18). The experiments *in vivo* and *in vitro* reported in this paper clearly show that tamoxifen, an antiestrogenic agent, strongly inhibits hepatocyte proliferation. This inhibition is maximal when tamoxifen is injected *in vivo* immediately after the hepatectomy and *in vitro* when the drug is added to the cultures during the first 30 hr.

Our data raised three major questions which are related to the role that estrogen plays in hepatocyte proliferation: (i) Does tamoxifen affect EGF receptors in the liver? (ii) Is the inhibitory effect of tamoxifen mediated by the binding to the estrogen receptor? (iii) What is the mechanism of action of estrogen and tamoxifen on hepatocyte proliferation?

In regard to the first question, our data show that tamoxifen does not change the behavior of EGF receptors during regeneration; in fact, B_{max} and K_d were similar in rat livers 24 hr after hepatectomy, whether or not the rats had been treated with tamoxifen. This demonstrates that the tamoxifen effect is due to an inhibition of some intracellular biochemical reaction which might be independent of EGF receptor interactions. With respect to the second question regarding the binding of tamoxifen to liver estrogen receptors, it is likely that this is the case, since tamoxifen competes effectively for estradiol binding to estrogen receptor in human liver (33). The possibility that tamoxifen is interacting with anti-estrogen-binding proteins, recently demonstrated in microsomal fractions of the liver (34, 35), is very unlikely. These proteins differ from estrogen receptors by a different pH stability profile, a different sensitivity to protease and a typical ligand binding specificity. In addition, these proteins show a moderate affinity for compounds like tamoxifen which have antiestrogenic potencies. The results, *in vivo* and *in vitro*, reported in this paper strongly suggest that the inhibitory effect of tamoxifen is due to its ability to bind to estrogen receptors, since the inhibition *in vivo* is removed by the simultaneous administration of equimolar amounts of estradiol and *in vitro* the stimulatory effect of estrogen in the presence of 5% NRS is abolished by tamoxifen.

The third point is more difficult to answer. Our data show that in order to influence regeneration, tamoxifen has to be administered very early after hepatectomy. It

has recently been reported that tamoxifen is able to inhibit proliferation of different cell types by blocking the transition of the G_0/G_1 phase of the cell cycle (36–40). A reversal of this effect by estrogen has also been shown (36). Our *in vivo* and *in vitro* results indicate that the inhibitory effect of tamoxifen also occurs during the first stages of hepatocyte proliferation. The difference in sensitivity between *in vivo* (6 hr) and *in vitro* (36 hr) conditions reported by us is most likely due to a lack of synchrony in the hepatocyte cultures and to the difficulty in determining the true zero time for stimulation by mitogens. The results reported in Table 5 indicate that tamoxifen inhibits Na^+ influx, which is an early event in hepatocyte proliferation, according to Koch and Leffert (16). Probably as important is our finding, also shown in Table 5, that estrogen increases the Na^+ flux. The finding that Na^+ influx is increased when estrogen is added to hepatocyte cultures provides further evidence for the mitogenic properties of estrogen and also suggests that estrogen acts early in the progression of hepatocytes from a quiescent to a proliferative state. Therefore, other mechanisms by which tamoxifen may exert its inhibitory effect must also be considered. Although there is no evidence that the activation of protein kinase C is mitogenic for hepatocytes, it is possible that estrogen and tamoxifen modulate the expression of this protein (41, 42). Tamoxifen is also known to inhibit calmodulin (43) and the importance of calmodulin in cell proliferation is well known (44).

At present, it is difficult to reach a final conclusion on the complex effects of both estrogen and tamoxifen on hepatocyte proliferation. This report, however, provides additional evidence that estrogen has mitogenic properties for hepatocytes *in vivo* and *in vitro* and may play an important role in hepatocyte proliferation.

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