Androgen and Estrogen Receptors in Regenerating Male Rat Liver

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The main interest of many investigators is to studie the effect of hepatic resection in the remnant liver and identifie the mechanism that regulate the regenerative process.

During the last 10 years, many hormones (such as insulin, glucagon, epidermal growth factor (3,4,7,9,13,18,25,28), parathyroid hormone (26), calcitonin (18), iodothyronines (6) and glucocorticoids (8) have assumed a prominent role as factors related in some way to liver proliferation following partial hepatectomy (P.H.). However there are several lines of evidence suggesting that estrogens have a potential role in liver cell proliferation.

First, estrogen have been implicated in certain liver disease characterized by increased mitogenic activity (reviewed in references 11, 20). Furthermore, livers containing hepatic adenoma and focal nodular hyperplasia have demonstrated increased estrogen receptor (ER) content (23). Thus it was of interest to investigate potential influences of estrogens in regeneration;in a recent study our group reported changes in cytosolic and nuclear ERs in regenerating rat liver during the first 72 h.after hepatectomy (14). In this report we have determined,in 70% hepatectomized male rats, plasma levels of estrogen and androgen,cytosolic and nuclear hepatic receptors for both hormones,and alteration in two male specific metabolic markers (MEB and E2-OHase) which may reflect the activity of hormone receptor complex in the regenerating liver.

MATERIAL AND METHODS

Animals

Male Sprague-Dawley rats (250 g) used in these studies were maintainned on standard rat laboratory diet and water at libidum in a temperature and light-controlled room (6:30 AM-6:30 PM). Partial hepatectomy was performed according to the methods of Higgins and Anderson (17). All operations were performed between 7:30 and 9:00 AM using ketamine anesthesia (10 mg/100 g. b.wt.). In sham-operated animals used as 0 time controls, the liver was manipulated in the same manner as the livers of resected animals, and returned to the abdomen. The animals were killed by decapitation 12, 24, 48, 72, 96 and 144 hours after partial hepatectomy.

Materials

Estrone (E1), estradiol (E2), were purchased from Steraloids, Wilton, NH. Diethylstilbesterol (DES), leupeptin, bovine serum albumin, sodium molybdate, protamine sulfate and other buffer components were purchased from Sigma Chemical Company, St. Louis, MO. Norit A and dextran C were obtained from Fisher Scientific Company, Pittsburgh, PA. Radioactive (2, 4, 6, 7, 16, 17)- (3H)-estradiol, (3H)-E2, 151 Ci/mmol; (3H)-methyl s-adenosylmethionine, 10 Ci/mmol; (3H)-methyl thymidine, 77 Ci/mmole; 17 alfa methyl-(3H)-methyltrienolone (3H)-R1881 , 87 Ci/mmol; non radiolabeled R1881, and Econofluor scintillation fluid were obtained from New England Nuclear, Boston MA., Aqueous Counting Scintillant (ACS) was purchased from Amersham, Arlington Heights, IL.The radiolabeled steroids used in these studies were assayed periodically for purity by thin-layer chromatography on silica gel G in ethyl acetate/hexane/ethanol (85:10:5), and were used only if purity was 95%. The sources of other material heve been described (17, 10).

Buffers

Unless otherwise stated, all experiments were performed at 0-4 °C using the following buffers: 0.01M Tris HCl, 1.5 mM EDTA, pH 7.4 (TE buffer); TE buffer with 5 mM dithiothreitol (TED buffer); TE buffer with 20mM sodium molybdate (TEM buffer); TE buffer with 0.25M sucrose (TES buffer); with both of the latter additions (TEMS buffer) and 0.25M sucrose, 3mM MgCl2, 10mM HEPES, pH 7.4 (SMgH buffer) and SMgH with 20mM sodium molybdate, pH 7.4 (SMgHM buffer). Leupeptin (0.15 mM)and benzamidine (1.0 mM) were added to

all buffers used in preparation of nuclei and cytosol, and to those used for gel filtration chromatography.

Binding study

Cytosol receptors (cER and cAR). The protamine sulfate precipitate method was used to assay cytosolic estrogen receptor (cER); this method avoid interference of (3H)-E2 binding to the high-capacity estrogen binder (MEB) of male rat liver cytosol (17, 10). Cytosol and protamine sulfate precipitates thereof were prepared as described previously (17). The dextran-coated charcoal method (DCC) previously described (17,10) was used to assay cytosol androgen receptor (cAR).

All subcellular fractions were prepared from the same liver. Final protein concentration of the cytosol suspension was typically 25-30 mg/ml.

Cytosol prepared as described above was diluted with one volume of TEM buffer before use. All incubations for determination of androgen receptor included 500nM triamcinolone acetonide (TA) to block any contribution of the glucocorticoid receptor to (3H)-R1881 binding. The ligand commonly used in other tissues, (3H)-dihydrotestosterone, could not be used in liver because of extensive metabolism of this substrate; however no metabolism of (3H)-R1881 could be detected under these conditions (16, 12).

Nuclear receptors (nER and nAR). The exchange assay method was used to determine both estrogen and androgen receptor.

Nuclear Estrogen Receptor: Nuclei were prepared and washed as previously described (14). Cytosolic contamination of nuclei was assayed by determining the activity of alcohol dehydrogenase (30) in the homogenate, cytosol, and the washed nuclei preparation and was found to be less than 0.5% in the nuclear preparation. The average recovery of DNA in the nuclear preparation was 72.8% of that in the homogenate. Nuclear suspensions (0.2 ml) were incubated in the presence of 10 nM(3H)-E2 in SMgH buffer with and without a 100 fold excess concentration of unlabeled DES at 30 °C for 1 h, conditions previously determined to be optimal for the exchange assay (12). The bound steroid was extract from the nuclear pellet with 2 ml of absolute ethanol at 30 °C for 30 min. The ethanol extract was counted with 10 ml of ACS in a Packard Tri-Card spectrometer. The remaining pellet was used for DNA quantification.

Nuclear Androgen receptor: in order to determine the best concentration of steroid for a one point assay, aliquots (200 1) of the nuclear suspension from liver were incubated with 0.2-10nM

(3H)-R1881 and 1 μM TA at 4 °C overnight in the absence (total binding) and presence (nonspecific binding) of a 100-fold excess of unlabeled R1881. The optimum conditions proved to be an incubation with 5nM(3H)-R1881; these conditions minimized nonspecific binding while specific binding was saturating. The washed pellet was extracted with 2 ml of ethanol for 1h at 30 °C; the entire pellet and extract were transferred to a 20 ml scintillation vial and 8 ml of ACS scintillation fluid was added.

Other methods

Assay of Androgen-Responsive Hepatic protein: Assays for the determination of cytosolic content of a male specific estrogen binder (MEB) and of microsomal estrogen 2-hydroxylase (E2-OHase) activity has been previously described (29).

Labeled nuclei and DNA synthesis were estimated on the 1st, 2nd, 3rd, 4th, 6th days after partial hepatectomy (14,22,23), DNA content was measured by the diphenylamine method of Burton (5) using calf thymus DNA as standard.

Protein concentrations were determined by the method of Lowry et al (19), Plasma testosterone and estradiol were determined by specific RIAs as previously described (31). Equilibrium dissociation constant (Kds) and the concentration of binding sites were calculated by the method of Scatchard (27). Linear regression analysis of Scatchard plots was performed on a Texas Instrument T155 calculator. Statistical analysis were performed using Student's t-test program available on the Hewlett Packard 9815S.

RESULTS

Figure 1 displays the content of estrogen receptors (panel A-B-C), as well as androgen receptors (panel D-E-F) of rat liver at different times after 70% hepatectomy. cER decreases rapidly during the first 12 h (0.3 pmol/g of liver) and remains near this level until 48 h after hepatectomy when the lowest recorded cER is reached (0.24 ± 0.02 pmol/g of liver). The values for cER at 12,24 and 48 h are significantly lower ($p<.005$) than that at 0 h. After this time the level of cER returns to somewhat greater than normal and than becomes normal for the remainder of the observation period (0.63 pmol/g of liver ± 0.06 pmol/g of liver). The affinity of cER for estrogen did not vary as a function of the change of receptor level. The Kd values for cER at all time points studied were similar (0.5-2.5 nM). The level of nER, in contrast, after a

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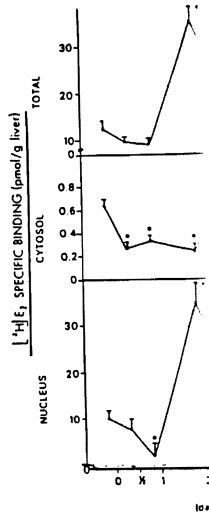

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small decrease during the first 24 h following hepatectomy increases rapidly reaching the zenith at 48 h (35 pmol/g of liver weight), significantly different ($p < 0.005$) than the value at 0 h. After this period, beginning at 72 h, the hepatic content of nER slowly returns to normal values (10 ± 1 pmol/g of liver weight). The total hepatic estrogen receptor (ER) shown in panel A parallels the increase in nER.

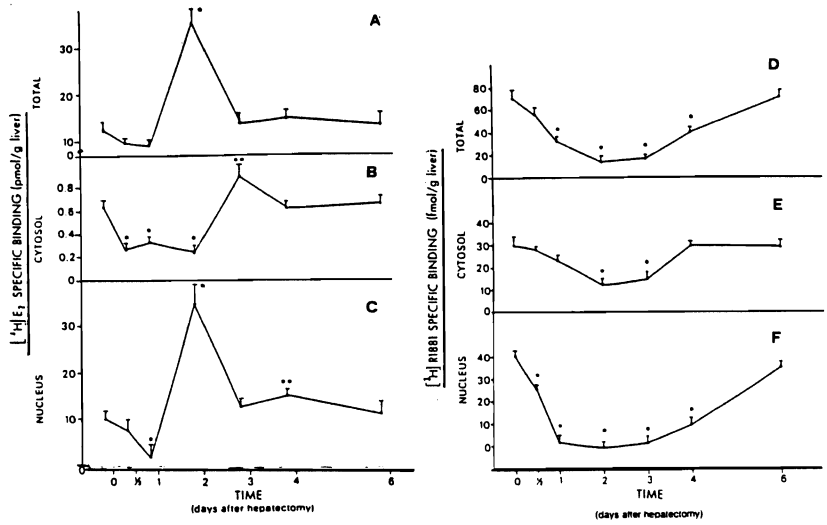


Fig.1: Variations in sham operated (0 time) and hepatectomized rats (days after partial hepatectomy) in specific (3H)-E2 (A-B-C) and (3H)-R1881 (D-E-F) binding. The values are expressed as M±S.D. Significantly different from the 0 time value (*=$p<0.005$; **= $p<0,01$).

Different is the panel shown by the hepatic content of AR at different times after 70% hepatectomy: cAR levels (panel E) decreased slowly, beginning 12 h following hepatectomy and reaching minimum levels at 48 h (10 fmol/g of liver). After this time the value returns to normal at 4 days after the hepatectomy (30 fmol/g of liver), remaining stable at this level during the remainder of the observation period. At no time studied was a variation detected in cAR affinity for androgen. Kd values did not vary, having a mean value of (0.55-1.8nM). The values of nAR present a similar picture. In fact, they decrease rapidly during the first day following hepatectomy and reach a value near zero (2.0 ± .02 fmol/mg), remaining at this level for the next 48 h.

Beginning at 72 h the nAR rises, returning to the normal value 6 days after hepatectomy. As a result of this change, the total binding capacity of hepatic androgen receptor (panel D) is extremely reduced, especially during the time of maximum rate of DNA synthesis (3H-Thymidine incorporation) and labeled nuclei (Fig. 2)

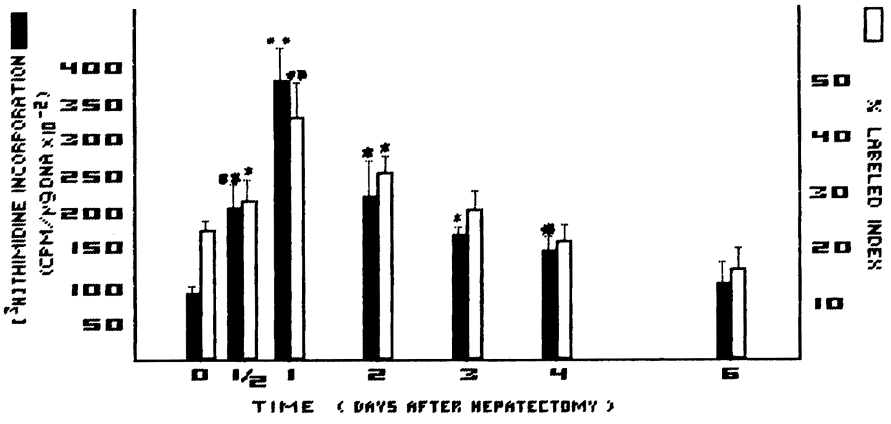
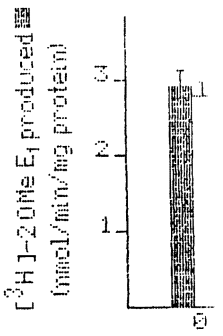


Fig. 2. DNA synthesis and labeled index after partial hepatectomy. DNA synthesis was determined as reported in Material and Methods, and expressed as (3H)-thymidine incorporation/ g DNA at different times after 70% partial hepatectomy (solid bars). Labeled index is expressed as the number of labeled nuclei for 100 cells, as outlined in Materials and Methods. The values are expressed as M±S.D. Significantly different from sham operated (0 time) animals are indicated (*=p <0.05; **=p <0.001).

Figure 3 illustrates the effect of regeneration on the level of two androgen responsive proteins. The activity of the microsomal enzyme E2-OHase, displays a time dependent decline during regeneration. On day 1 posthepatectomy, the activity of E2-OHase is essencially normal, but decreases drastically on day 2 to 13% of normal values and does not rise appreciably over the next 4 days. In addition the level of a second androgen responsive protein, a male specific estrogen binder (MEB), rapidly declines to about 15% of normal values within 24 h after partial hepatectomy. The level of MEB rises gradually during the next five days posthepatectomy, but does not reach normal levels even by day 6.



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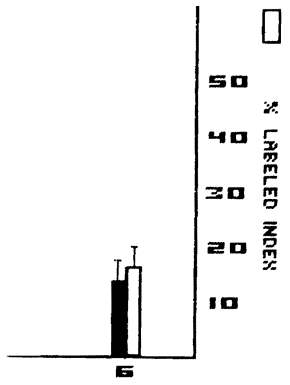

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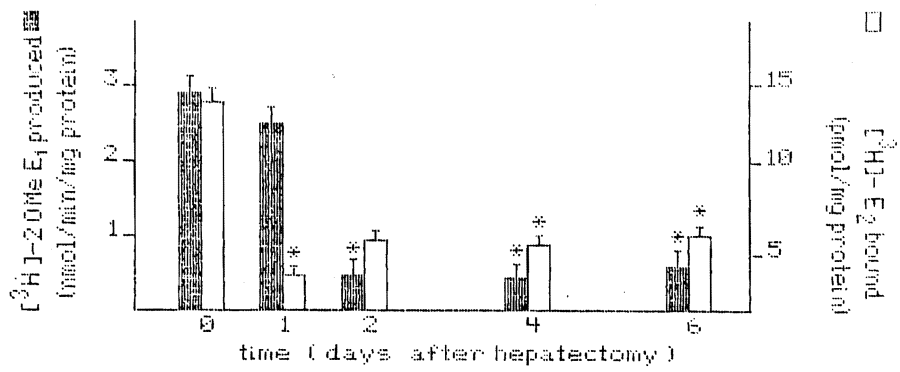

Fig. 3. Activity of two androgen responsive proteins after partial hepatectomy. Microsomal E2-OHase (solid bars) activity was measured as the amount of (3H)-20MeE1 product formed, and cytosolic MEB was quantitated by its (3H)-E2 binding activity, as described in Methods. All groups consisted of at least three animals except for the MEB determination on day 2 which consisted of a single rat. The values are expressed as M±S.D. Significantly different from the sham operated animals (0 time) (*= $p < 0.005$).

Interestingly are also the variations of the serum sexual hormones (tab. 1); the testosterone level decrease during the same period when the total hepatic binding capacity for androgen is greatly reduced; in contrast serum estradiol rises at 1 day after surgery and this is significantly ($p < 0.005$) at 3 days.

	days after hepatectomy						
HORMONES	0	1/2	1	2	3	4	6
E^2 pg/ml	6.66 ± 3.33	6.66 ± 4.16	5.83 ± 3.33	13.33 ± 6.66	26.66 ± 10.00 p<0.005	5.83 ± 1.66	5.00 ± 3.33
TESTOSTERON ng/ml	1.6 ± 0.45	0.63 ± 0.13 p<0.005	0.27 ± 0.18 p<0.005	0.18 ± 0.18 p<0.005	0.63 ± 0.45 p<0.005	1.18 ± 0.36	1.36 ± 0.27

DISCUSSION

The data reported herein show significant variations in cytosolic and nuclear receptor for androgen and estrogen during the course of liver regeneration after 70% hepatectomy.

The receptor redistribution starts at the same time as the stimulation of DNA synthesis and reaches its maximum at 48 hours coinciding with the high value of labeled index. From the present data we do not know whether the increase in estrogen receptor content represents de novo synthesis of receptor or an activation of existing receptors and we cannot draw a cause and effect relationship between this increase and hepatic regeneration after 70% hepatectomy, but the data is suggestive of a critical role for estrogens and their receptors in the regenerative process.

Even more obscure is the apparent failure of the androgen responsive mechanisms of the liver. The decrease in the levels of the androgen receptor and the two androgen responsive proteins is remarkable in that these changes occur within 24-48 hours after surgery and parallel the decrease in serum testosterone. The decrease of androgen and the corresponding failure of androgen receptor and androgen dependent proteins in the liver could be due to a hormonal interaction not yet understood, such as a decreased synthesis or an increased clearance of testosterone. On the other hand, the hepatic androgen system failure might be a response to the increase in serum estrogen, or an increased interaction of estrogen with its receptor. These possibilities are currently under investigation.

In light of the dramatic decrease in androgen mediated hepatic responses, one may speculate whether the increase in nuclear estrogen receptor might be important in the stimulation, cell division or induction of specific proteins necessary for this regeneration. Estrogen, in fact, is a known inducer of the hepatic synthesis of plasma transport proteins (1), a function likely to be critical during regeneration. The presence of a massive estrogenic effect during hepatocyte proliferation gives the impression of a return to a fetal phenotype expression of the liver, opening the question of whether or not these compounds might be implicated with the process of carcinogenesis. Since Baum (2) in 1970 first reported the possible relationship between estrogen therapy and hepatic tumors, many disorders in human such as hepatic carcinoma and hyperplasia have been attributed to estrogen therapies prescribed for a variety of clinical reasons (23).

The effect of estrogen in promoting diethylnitrosamine-induced liver tumors has been addressed recently by Wanless et al (32).

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These studies have shown that estrogens promote the development of hepatic neoplasms associated with increasing hepatocyte mitogenetic activity. In contrast, Mishkin et al (21) has demonstrated that in animals with acetylaminofluorine-induced hepatic hyperplastic nodules, estradiol in combination with tamoxifen resulted in regression of the nodules. In a recent study, Porter et al (24) found an increase of nuclear and total estrogen receptor activity in the liver of human females with focal nodular hyperplasia occuring in association with oral contraceptive use; a increase in nuclear estrogen binding activity was noted in hepatic adenoma. In addition, we (15) recently reported a very low level of cytosolic estrogen binding activity in Morris hepatoma 7777 suggesting that the decrease of cytosol receptor in neoplastic tissue could be an expression of active nuclear translocation, or a loss of receptor as a function of dedifferentiation of the tissue.

The results reported here allow another consideration regarding the relationship between androgen and estrogen in the liver. If the failure of androgen related systems is linked to the increase of estrogen related livers system, as appears to happen during the first 72 h after hepatectomy, hepatocyte proliferation, and perhaps hepatic carcinogenesis could occur in the male whenever the androgen related systems fail due to interaction of carcinogenic substances with the liver. Under these circumstances, the estrogen receptor could increase in such males, permitting the possibility of an increased potential for the induction of estrogen mediated liver diseases.

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