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Effect of Antiandrogen Flutamide on Measures of Hepatic Regeneration in Rats

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Male rat liver undergoes a process of demasculinization during hepatic regeneration following partial hepatectomy. The possibility that antiandrogens might potentiate this demasculinization process and in so doing augment the hepatic regenerative response was investigated. Adult male Wistar rats were treated with the antiandrogen flutamide (2 mg/rat/day or 5 mg/rat/day subcutaneously) or vehicle for three days prior to and daily after a 70% partial hepatectomy. At various times after hepatectomy, the liver remnants were removed and weighed. Rates of DNA and polyamine synthesis were assessed by measuring thymidine kinase and ornithine decarboxylase activities, respectively. Hepatic estrogen receptor status and the activity of alcohol dehydrogenase, an androgen-sensitive protein, were measured. Prior to surgery, the administration of 5 mg/day flutamide reduced the hepatic cytosolic androgen receptor activity by 98% and hepatic cytosolic estrogen receptor content by 92% compared to that present in vehicle-treated controls. After hepatectomy, however, all differences in sex hormone receptor activity between the treatment groups were abolished. The rate of liver growth after partial hepatectomy in the three groups was identical. Moreover, hepatectomy-induced increases in ornithine decarboxylase activity and thymidine kinase activity were comparable. These data demonstrate that, although flutamide administration initially alters the sex hormone receptor status of the liver, these affects have no effect on the hepatic regenerative response following a partial hepatectomy.

KEY WORDS: antiandrogens; hepatic regeneration; hormone receptors; androgens.

The liver of mammals has been found to have a number of sexually dimorphic characteristics (1–3). During regeneration following partial hepatectomy, male rat liver is demasculinized in that many male-specific hepatic attributes disappear with the onset of the regenerative response (4). These changes include increases in hepatic estrogen receptor activity and serum estradiol levels and reductions in the level of serum testosterone and the hepatic content of androgen receptor, as well as reductions in the levels of several androgen-responsive hepatic proteins. The importance of hepatic demasculinization during regeneration has not yet been determined. However, if hepatic demasculinization is an important aspect of the regeneration process, then the administration of an antiandrogenic drug might be expected to intensity the demasculinization process and in so doing increase the rate of regeneration.

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To investigate this hypothesis and the role of androgens in hepatic regeneration, the antiandrogen flutamide (a,a,a-trifluoro-2-methyl-4′-nitro-*m*-propionotoluidide) was administered to adult male rats and the regenerative response following partial hepatectomy was examined. Flutamide is a nonsteroidal antiandrogen that has been shown to have little or no intrinsic androgenic activity (5, 6).

Following a standard two-thirds hepatectomy, the rate of growth of the liver remnant was determined both in flutamide-treated animals and vehicle-treated controls. In addition, the hepatic activities of thymidine kinase and ornithine decarboxylase were determined to assess the rates of DNA and polyamine synthesis, respectively. The activity of the estrogen receptors within the liver was determined to monitor the effect of treatment upon the estrogen receptor status of the liver during the regenerative process. Finally, the activity of hepatic alcohol dehydrogenase, an enzyme whose activity is repressed by androgens (7, 8), was assayed.

MATERIALS AND METHODS

Animals and Supplies. Adult male inbred Wistar rats (200–350 g) were purchased from Harlan Sprague Dawley (Indianapolis, Indiana). The flutamide used was a generous gift of Schering Co. (Bloomfield, New Jersey). New England Nuclear (Boston, Massachusetts) was the source for the [^{14}C]ornithine (57.6 mCi/mmol), [^{3}H]estradiol (99 Ci/mmol), [^{3}H]R1881 (87 Ci/mmol), and unlabeled R1881 utilized. Tritiated thymidine (5 Ci/mmol) and ACS scintillation fluid were purchased from Amersham (Arlington Heights, Illinois). Absolute ethanol and DEAE-cellulose paper were purchased from U.S. Industrial Chemicals Co. (Tuscola, Illinois) and BioRad (Richmond, California), respectively. Unlabeled ornithine, pyridoxal phosphate, Tris base, diethylstilbestrol, adenosine triphosphate, sodium molybdate, nicotinamide adenine dinucleotide, calf thymus DNA, and bovine serum albumin were purchased from Sigma Chemical Co (St. Louis, Missouri). All other chemicals were purchased from Fisher Chemical Co (Pittsburgh, Pennsylvania).

Animal Treatment and Tissue Preparation. Male Wistar rats were assigned randomly to the various treatment groups. Animals in the flutamide-treated groups were given either 2 mg or 5 mg flutamide dissolved in a vehicle consisting of 0.2 ml peanut oil–acetone (9:1) subcutaneously beginning 72 hr prior to partial hepatectomy and every 24 hr thereafter until the time of sacrifice. Animals used as placebo controls received 0.2 ml of the peanut oil–acetone vehicle administered subcutaneously using the identical dosing schedule. A two-thirds partial hepatectomy was performed under light ether anesthesia as described by Higgins and Anderson between 9:00 and 12:00 AM (9). There was no difference in surgical mortality between the treatment groups. The liver tissue removed at the time of hepatectomy served as the zero time control sample for each animal and was used to differentiate between the effects of drug treatment and hepatic resection for the various parameters assessed.

At various times up to 72 hr after hepatectomy, animals from the vehicle control group and the two flutamide-treated groups were anesthetized with ether and weighed. The liver remnant was removed, weighed, and homogenized in 4 vol of ice-cold buffer consisting of 0.25 M sucrose, 1.5 mM EDTA, 10 mM mercaptoethanol and 10 mM Tris HCl (pH 7.4) using a Brinkman Polytron homogenizer. Cytosol was prepared by centrifugation at 103,000*g* for 1 hr at 4°C. All cytosolic enzyme assays were performed immediately after preparation of the cytosol.

Hepatic nuclear samples were prepared as described by Porter et al. (10). Briefly, the livers were homogenized in 3 vol of SMH buffer, consisting of 0.25 M sucrose, 3 mM $MgCl_2$ in 10 mM HEPES (pH 7.4), with the addition of protease inhibitors (10 mM benzamidine, 0.15 mM leupeptin, 0.25 mM pepstatin A, and 0.5 mM phenylmethylsulfonyl fluoride). Following centrifugation at 800*g* for 15 min, the pellet was resuspended using a Dounce homogenizer in SMH buffer containing the protease inhibitors and 0.15% Triton X-100. The samples were centrifuged at 800*g*, and the final pellet was suspended in SMH buffer.

Ornithine Decarboxylase Assay. Ornithine decarboxylase activity was determined by measuring the $^{14}CO_2$ released from labeled ornithine (11). In this assay, 0.4 ml cytosol was preincubated for 5 min at 37° C with a mixture containing 0.2 mM pyridoxal phosphate, 5 mM dithiothreitol, 1.5 mM *l*-ornithine in 10 mM Tris HCl (pH 8.0). At the end of this period, 0.5 μCi [1-^{14}C]*dl*-ornithine was added to the mixture and 250 μl of ethanolamine–ethylene glycol (2:1) was added to a center well to act as a CO_2 trap. The assay flask was sealed and incubated at 37° C for 1 hr. The reaction was terminated by the injection of 0.1 ml saturated trichloroacetic acid solution into the reaction mixture. After maintaining the reaction flask at 37° C for an additional hour, the CO_2 trapping solution was removed and placed into a glass scintillation vial containing 10 ml ACS scintillation fluid. Radioactivity was measured in a Packard Tri-Carb 460 CD liquid scintillation system (Downers Grove, Illinois).

Thymidine Kinase Assay. Thymidine kinase activity was determined by measuring the *in vitro* conversion of thymidine to thymidine phosphate (12). Cytosol (0.1 ml) was mixed with 850 μl of incubation buffer, consisting of 5 mM adenosine triphosphate, 3.6 mM $MgCl_2$ in 50 mM Tris HCl (pH 8.0), and 50 μl 1 μM [^{3}H]thymidine. The reaction was maintained at 37° C for 10 min and then terminated by immersion in boiling water for 2 min. After cooling in an ice bath, denatured protein was removed by centrifugation at 1500*g* for 5 min at 4° C. An aliquot (0.1 ml) of the supernatant was then spotted on a 3.8 × 3.8-cm piece of DEAE-cellulose paper. The paper was washed twice with 1 mM ammonium formate for 5 min followed by distilled water for 3 min. Next, the paper was placed in a glass scintillation vial, and the radioactivity bound to the paper was eluted into solution by the addition of 1 ml 0.1 M HCl–0.2 M KCl. After 15 min, 10 ml ACS scintil-

lation fluid was added, and ^3H present in the vial was determined.

Sex Steroid Receptor Assays. Evidence indicating that estrogen receptors and, by inference androgen receptors, may actually be located in the nucleus only has been published (13, 14). Currently the traditional terms "cytosolic receptors" and "nuclear receptors" are considered to refer to ligand-free receptors loosely bound to nuclei and to occupied receptors tightly bound to nuclei, respectively. Since the actual subcellular localization of the receptors was not pertinent to the present study, the traditional nomenclature has been employed.

The activity of the cytosolic estrogen receptors was determined by measuring the specific binding of a saturating concentration of [^{3}H]estradiol (15). Hepatic cytosol prepared as described above was diluted 1:1 with buffer consisting of 40 mM sodium molybdate, 1.5 mM EDTA, and 10 mM Tris HCl (pH 7.4) to stabilize the receptors. To measure total binding of the ligand, 200 μl of diluted cytosol was mixed with 25 μl 30 nM radioactive ligand and 25 μl ethanol. Nonspecific binding was measured in parallel assay in which the ethanol was replaced with 25 μl 3 μM unlabeled DES dissolved in ethanol. Male-specific estrogen binding protein binding was blocked by the inclusion of 200 μM 2-methoxyestriol in all cytosolic estrogen receptor assays (25). After 2 hr at 4° C, the incubation was terminated by the addition of 0.4 ml 1% dextran-coated charcoal to each tube to remove unbound ligand (15). This suspension was then centrifuged for 5 min at 1500*g* at 4° C, and the supernatant was carefully transferred to a scintillation vial containing 8 ml ACS scintillation fluid. The radioactivity in the vial was measured.

Nuclear estrogen receptor levels were measured by the method of Porter et al (10). Aliquots of the hepatic nuclear preparation were incubated in parallel at 30° C for 90 min with 5 nM [^{3}H]estradiol in the absence and presence of a 200-fold excess of unlabeled diethylstilbestrol to measure total and nonspecific binding, respectively. At the end of the incubation period, unbound ligand was removed by four successive washings of the nuclei with ice-cold SMH buffer. After washing, absolute ethanol was added to the nuclear pellets and the tubes were maintained at 30° C for 30 min to facilitate the extraction of bound ligand. After centrifugation at 1600*g* for 10 min, the supernatant was pipetted into scintillation vials and the radioactivity was measured.

The cytosolic androgen receptor assay used was similar in design to that described above for the cytosolic estrogen receptor assay (4). Tritiated R1881, a synthetic androgen, was used as a labeled ligand and unlabeled R1881 was used in the nonspecific binding assays. Triamcinolone acetonide (5 μM) was included in all androgen receptor assays to block binding of R1881 to glucocorticoid receptors. The only other difference between the two assay methods is that in the androgen receptor assay, the cytosol was incubated overnight at 4° C with the ligand rather than for 2 hr as in the cytosolic estrogen receptor assay.

Alcohol Dehydrogenase Assay. Alcohol dehydrogenase activity was measured spectrophotometrically at 340 nm at ambient temperature by measuring NADH production

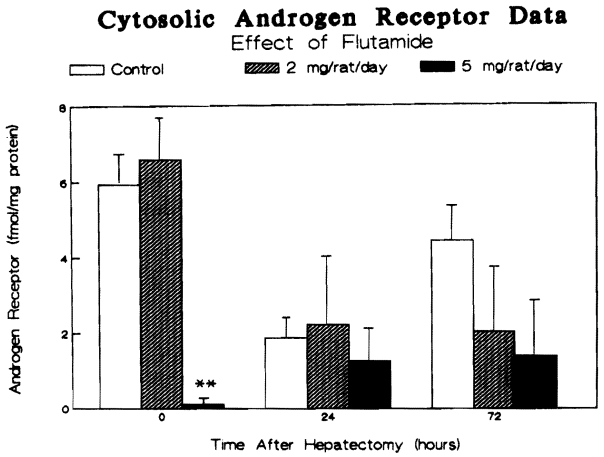

Fig 1. Effect of flutamide administration on hepatic cytosolic androgen receptor activity during hepatic regeneration. Receptor activities are reported as means ± SEM for 3–11 animals per group. $^{**}P < 0.01$ vs vehicle-treated controls.

in a 1-ml reaction mixture consisting of cytosol, 2.8 mM nicotinamide adenine dinucleotide, and 18 mM ethanol in 100 mM Tris HCl buffer (pH 7.2) (16).

Miscellaneous Methods and Procedures. Protein concentrations were determined by the method of Lowry et al (17) with bovine serum albumin being used as the standard. DNA was measured fluorometrically (18) using calf thymus DNA as the standard. Statistical analysis of the data were performed using one-way ANOVA followed by Scheffe's test through the Abstat program on an IBM PC-XT. A *P* value of 0.05 or less was considered to represent a significant difference. All results are presented as mean values ± SEM.

RESULTS

Pretreatment with the higher dose flutamide prior to partial hepatectomy reduced the hepatic androgen receptor content to barely detectable levels (Figure 1). In the liver of the control animals and the animals treated with the lower dose of flutamide, partial hepatectomy resulted in a rapid decrease in the cytosolic androgen receptor content at 24 hr, with a return toward normal levels at 72 hr following surgery (Figure 1).

The effects of flutamide treatment on changes in hepatic cytosolic estrogen receptor activity were similar in pattern to those observed for the androgen receptor and are presented in Figure 2. The zero time hepatic cytosolic estrogen receptor activity of animals pretreated with the higher dose of flutamide was lower than that of the rats receiving either the lower dosage of the drug or the vehicle. Partial hepatectomy resulted in a reduction in the cytosolic activity of the estrogen receptors at 24 hr following surgery in the control animals. At 72 hr

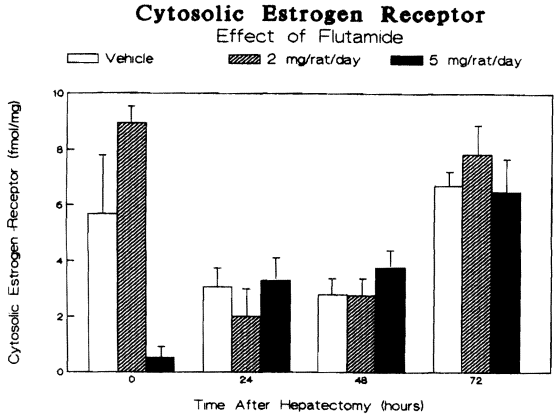

Fig 2. Effect of flutamide administration on hepatic cytosolic estrogen receptor activity during hepatic regeneration. Receptor activity is reported as mean femtomoles [^{3}H]estradiol bound per milligram cytosolic protein ± SEM for 3–11 animals per group.

following hepatectomy, all three groups showed a reduced hepatic cytosolic content of estrogen receptors compared to that of the vehicle controls at time zero, and no subsequent difference in the content of estrogen receptor activity of the hepatic cytosol between groups was observed. As presented in Figure 3, the hepatic nuclear estrogen receptor activity in control animals was increased threefold at 72 hr, a time when serum estradiol levels have been shown to be maximally increased in animals subjected to partial hepatectomy (4). Notably, the estrogen receptor content of the liver of the 5 mg/day-treated animals, both prior to surgery and at all subsequent time points, was not different from that of the vehicle-treated controls (Figure 3).

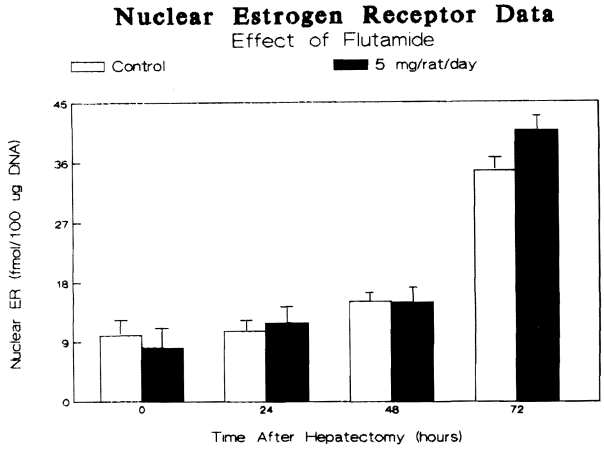

Fig 3. Effect of flutamide administration on hepatic nuclear estrogen receptor activity during hepatic regeneration. Data are presented as femtomoles [^{3}H]estradiol bound per 100 μg DNA and are means ± SEM for 3–11 animals per group.

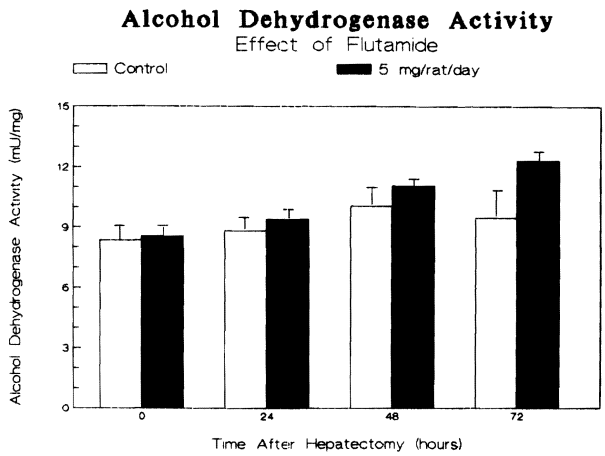

Fig 4. Effect of flutamide administration on hepatic alcohol dehydrogenase activity during hepatic regeneration. Alcohol dehydrogenase activities are presented as milliunits activity per milligram cytosolic protein and are means ± SEM for 3–11 animals per group. $^{**}P < 0.01$ vs vehicle-treated controls.

The effect of flutamide administration on the alcohol dehydrogenase activity of the liver during hepatic regeneration is shown in Figure 4. Neither partial hepatectomy nor treatment with flutamide significantly altered the activity of the enzyme within the liver at any time point as compared to that of the vehicle-treated controls.

Because ornithine decarboxylase is the rate-limiting step in polyamine synthesis (19), the effect of flutamide treatment on the induction of the activity of this enzyme was measured. The data presented in Figure 5 demonstrate that partial hepatectomy resulted in a 10-fold increase in the activ-

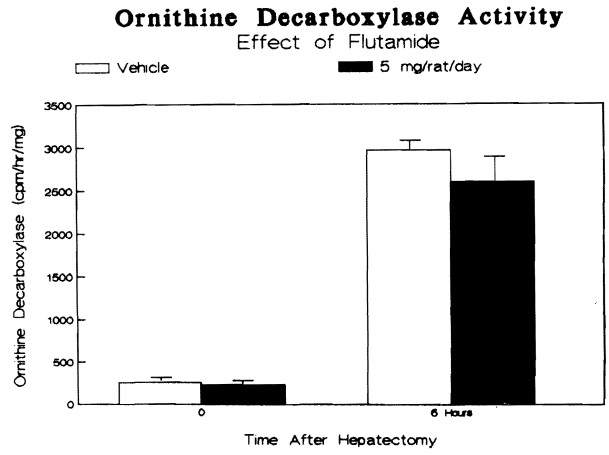

Fig 5. Effect of flutamide administration on the induction of hepatic ornithine decarboxylase activity after partial hepatectomy. Ornithine decarboxylase (ODC) activities are presented as counts per minute $^{14}CO_2$ released per hour per milligram cytosolic protein and are means ± SEM for 3–11 animals per group.

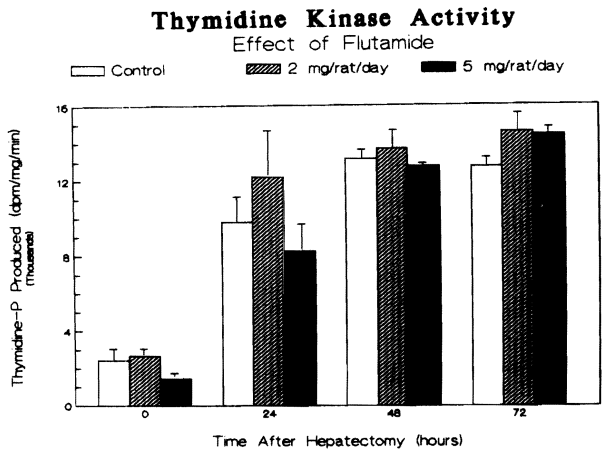

Fig 6. Effect of flutamide administration on hepatic thymidine kinase activity during hepatic regeneration. Thymidine kinase activities are reported as disintegrations per minute thymidine phosphate produced per minute per milligram cytosolic protein and are means ± SEM for 3–11 animals per group.

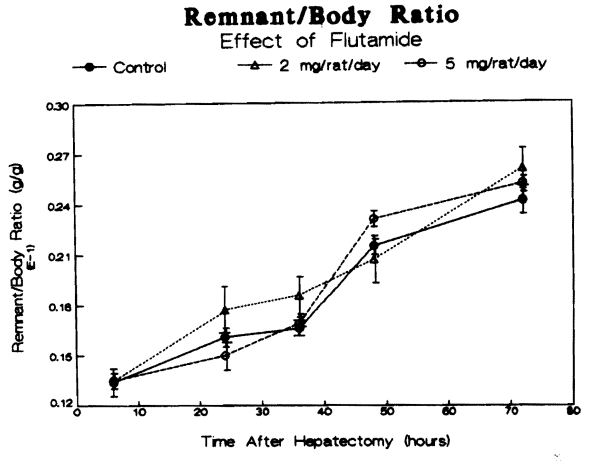

Fig 7. Effect of flutamide administration on liver growth after partial hepatectomy. Male Wistar rats were treated for three days prior to and daily after a two-thirds partial hepatectomy. The data are presented as ratios of remnant weight to body weight at the time of sacrifice and are means ± SEM for 3–11 animals per group.

ity of this enzyme in hepatic cytosol. More importantly, it shows that flutamide administration had no effect on the hepatic activity of ornithine decarboxylase either prior to or 6 hr after partial hepatectomy, the time at which the enzyme is induced maximally in response to the hepatectomy stimulus (20).

Hepatic regeneration necessarily involves an increase in DNA synthesis within the liver, which requires an induction of hepatic thymidine kinase activity (12, 21). In the present study, the activity of thymidine kinase at the time of hepatectomy was comparable in the three sets of animals studied (Figure 6, time zero). Within 24 hr following partial hepatectomy, a significant induction of thymidine kinase activity within the liver was observed in all treatment groups. Moreover, the magnitude of the induction was similar for all groups at each of the time points investigated (Figure 6).

The growth of the liver remnant after partial hepatectomy in control animals and the effect of the administration of the two dosages of flutamide on the rate of liver growth following two-thirds hepatectomy is presented in Figure 7. An approximately twofold increase in the liver–body ratio in the control animals was observed between 6 and 72 hr after hepatectomy. There was no difference in the rate of hepatic growth between either group of animals treated with flutamide and the vehicle-treated controls.

To further ensure that the increases in liver weight measured during the course of the experiments were not due either to hepatic glycogen accumulation, steatosis, or edema, the cytosolic protein concentration of the liver remnants was assessed (Figure 8). A transient, but probably not biologically important, difference ($P < 0.05$) between the 2 mg/day-treated animals compared to either the vehicle-treated controls or 5 mg/day drug-treated animals was observed 36 hr but not 72 hr after surgery.

DISCUSSION

The liver of male rats loses many of its male-specific characteristics during the regeneration that

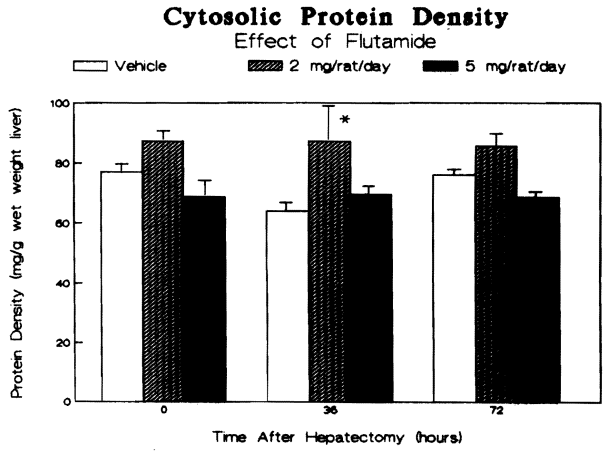

Fig 8. Effect of flutamide administration on hepatic cytosolic protein density during hepatic regeneration. Data are presented as milligrams cytosolic protein per gram wet weight liver and are means ± SEM for 3–11 animals per group. *$p < 0.05$ vs vehicle-treated controls.

occurs following partial hepatectomy (4). It was therefore hypothesized that a disruption of the androgen–estrogen balance might alter the rate of hepatic growth occurring after a two-thirds hepatectomy. In the present study the antiandrogen flutamide was administered to rats for three days in an attempt to demasculinize the liver and thereby possibly increase the regenerative response after partial hepatectomy. The effect of this treatment on hepatic growth and several other aspects of hepatic regeneration was then assessed.

Despite the fact that the doses of flutamide used here have been shown to alter the activity of certain sexually dimorphic hepatic enzymes (22) and did alter, at least at the higher dose used, androgen receptor levels prior to the partial hepatectomy stimulus for regeneration, in the present study relatively few effects of flutamide treatment were observed either prior to or following partial hepatectomy. Androgens have been shown previously to play a role in the growth of some cancers including hepatomas, and antiandrogen therapy has been useful in the treatment of some tumors, particularly prostatic cancers (23). Moreover, recently the use of a synthetic androgen has been shown to enhance the long-term survival of alcoholics with alcoholic hepatitis in a large multicenter cooperative study within the Veterans Administration (24).

Of the parameters investigated, the only statistically significant difference between the three groups studied prior to surgery was a 98% reduction in the hepatic cytosolic androgen receptor concentration in the 5 mg/day-treated animals, as expected, compared to that present in the other two groups. This reduction in hepatic androgen receptor content was anticipated on the basis of several previous studies. First, flutamide treatment interferes with the expression, activity, and recycling of the androgen receptor *in vivo*. Such influences have been noted for the estrogen receptor when an antiestrogen has been used (25). The binding of flutamide and its active metabolite flutamide-OH (a,a,a-trifluoro-2-methyl-4′-nitrolactotoluidide) to receptors in a variety of animal tissues had been investigated previously. The binding constant of flutamide for the androgen receptor varies, depending on the specific tissue being investigated, and ranges from 0.01 to 0.2% of the binding of testosterone to androgen receptors in the same tissue (26). The affinity of its major metabolite, flutamide-OH (27), for the androgen receptor is also tissue-specific and has a 7- to 80-fold greater affinity for the receptor compared to flutamide (26). Moreover, flutamide-OH has been shown to be an effective competitor for hepatic androgen receptors in *in vitro* binding assays (P.K. Eagon, unpublished observations).

Second, the administration of flutamide inhibits the hypothalamic–pituitary–gonadal axis and effects a reduction in testosterone synthesis. Testosterone is critical for the maintenance of androgen receptor activity, since castration of male rats was shown to reduce androgen receptor levels in the liver (28) and other tissues (29). Moreover, experiments in tissue culture of rat prostate and hamster ductus deferens cell lines (30) and human genital skin fibroblasts (31) have demonstrated that the cytosolic concentration of the androgen receptors is increased by the addition of androgens to the media. This up-regulation of the androgen receptor by its ligand is similar to the system hypothesized for the estrogen receptor (32). The antiandrogenic flutamide, or a metabolite thereof, could interfere either with gonadotropin secretion or androgen production and thereby reduce the level of androgen receptor within the liver (4).

The rate at which the androgen receptor levels responded to hepatectomy in this and previous (4) studies was much more rapid than the changes in receptor activity observed after castration. In other studies, hepatic cytosolic androgen receptor levels had required 8–10 days after castration for a 50% reduction in androgen receptor activity to be evident (28). Thus, the rapid reduction in androgen receptor levels seen after partial hepatectomy may involve factors other than a reduction in serum testosterone levels.

Further evidence that hepatic regeneration somehow alters the sex hormone regulatory system of the liver was provided by the changes observed in the hepatic alcohol dehydrogenase activity following partial hepatectomy. Previously, administration of androgens had been shown to reduce the activity of this enzyme in the liver by increasing the rate of degradation of the enzyme (33). Interestingly, in the present study the level of alcohol dehydrogenase activity in the control rats did not change during the course of the experiment.

Based on these data, it appears that endogenous regulatory factors induced by hepatic regeneration are more potent than flutamide in the regulation of the sex hormone receptor activity and its expression within the liver. The mechanism(s) by which hepatic regeneration alters sex hormone receptor activity within the liver is as yet uncertain. One

possible explanation is that the changes in sex hormone metabolism caused by the hepatectomy and the obligate loss of hepatic metabolic capacity result in alterations in the amounts of sex hormones available for binding with or regulation of the receptors at the level of the genome.

Another hypothesis involves the putative growth-stimulating factors released into the serum by regenerating liver tissue (34, 35). These agents could return to the liver and indirectly alter sex hormone receptor regulation within the liver. No data have yet been published examining the influence of such factors on the hepatic sex steroid receptor system. Alternatively, these liver-derived serum factors could alter some other system that could then exert some influence on the androgen and estrogen receptors. It is well established that modulation of the hypothalamic–pituitary axis alters the level of hepatic estrogen- and androgen-sensitive proteins (36, 37). The data obtained from the present studies do not allow a discrimination between these different possibilities.

These data are consistent, however, with previous studies (22) in which flutamide administration has been shown to affect the balance between androgenic and estrogenic influences on the liver. The hypothesis that these effects would increase the rate of hepatic regeneration, however, was not substantiated, whether the results were expressed in terms of the rate of liver growth, induction of polyamine synthesis, or induction of DNA synthesis. The observation that factors associated with the process of hepatic regeneration override the effects of the antiandrogen flutamide on androgen receptor activity suggests that androgens and their receptors play little or no apparent role in the process of hepatic regeneration.

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