Digestive Diseases and Sciences, Vol. 35, No. 1 (January 1990), pp. 6–11

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Effect of Cyclosporine on Hepatic Cytosolic Estrogen and Androgen Receptor Levels Before and After Partial Hepatectomy

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Estrogen and androgen receptors within the liver have been reported to modulate the hepatic regenerative response to partial hepatectomy. Moreover, cyclosporine has several untoward effects that might occur as a consequence of alterations in sex hormone activity. To evaluate these questions the following experiments were performed. Estrogen and androgen receptors in cytosol were quantitated in livers of rats treated with cyclosporine or olive oil vehicle before and after partial hepatectomy or a sham operation. Ornithine decarboxylase activity and thymidine kinase activity were assessed as indices of hepatic regeneration. Preoperative levels of estrogen receptor activity in the hepatic cytosol were significantly greater in rats treated with cyclosporine as compared to vehicle treated controls ($P < 0.01$). In contrast, preoperative levels of androgen receptor activity in the cyclosporine-treated and vehicle-treated animals were similar. Following partial hepatectomy, a reduction in the activity of both sex hormone receptors in the hepatic cytosol was observed and was compatible with results described previously in normal animals. Unexpectedly the preoperative levels of ornithine decarboxylase ($P < 0.01$) and thymidine kinase activity ($P < 0.01$) were significantly greater in the rats treated with cyclosporine as compared to the vehicle treated controls. As expected, ornithine decarboxylase activity (at 6 hr) and thymidine kinase activity (at 24 hr) rose and peaked in response to a partial hepatectomy but were significantly greater ($P < 0.05$) in the rats treated with cyclosporine as compared to the vehicle. These results show that cyclosporine treatment causes an increase in the hepatic content of estrogen receptor activity that is associated with an enhanced potential for a regenerative response. These effects of cyclosporine treatment on the sex hormone receptor levels in liver may explain the mechanisms responsible for some of the untoward effects of treatment with this agent.

KEY WORDS: cyclosporine; estrogen; androgen; hepatectomy; cytosol.

The presence and properties of sex hormone receptors in the liver have been studied extensively (1). However, the precise function of these receptors within the liver is unknown. Nonetheless, in mammals certain functions of the liver are known to display a sexual dimorphism (2–4). Testosterone, present *in utero* and after puberty, appears to be the major determinant of the differences between the sexes in hepatic function.

Hepatic regeneration following a partial hepatectomy is associated with a "feminization" of the

Manuscript received October 13, 1988; accepted July 24, 1989.
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Supported by research grants from the Veterans Administration project grant AM 29961, and from the NIDDK AM 32556, and from the NIAAA AA06601.
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0163-2116/90/0100-0006$06.00/0

liver in male rats as manifested by an increase in the serum level of estradiol and the hepatic activity of estrogen receptors. Moreover a reduction in the serum level of testosterone and the activity of the androgen receptors within the liver occurs (1, 5). Translocation of estrogen receptors from the cytosol to the nucleus in liver subjected to a prior partial hepatectomy coincides with the initiation of DNA synthesis. These observations suggest an important role for nuclear estrogen and, particularly, an estrogen–DNA interaction in the initiation of the regenerative process (1, 5).

In recent years orthotopic transplantation of the liver has become accepted as a reasonable treatment for patients with end-stage liver disease (6). The criteria currently used in the selection of donors for liver transplantation include ABO blood group and size compatibility with the recipient. Currently, the sex of the donor is not taken into consideration. In view of the sexual dimorphic character of hepatic function and the changes in the sex hormone status of the liver after transplantation (especially if the donor is of the opposite sex), the choice of a particular donor sex may be important for the initial success of the transplant procedure as well as subsequent long-term normal function of the graft (7).

Cyclosporine is the immunosuppressive agent most frequently used in organ transplantation. Currently identified side effects of cyclosporine include hepatotoxicity, Leydig cell failure, and increased lanugo hair growth (6, 8). The pathogenesis of the increased hair growth associated with cyclosporine use has not been determined yet. In this study, the effect of cyclosporine on the estrogen and androgen receptor content of the liver, both before and after partial hepatectomy, was determined. The latter condition was examined as a model of the regenerative response that occurs following liver grafting but avoids the confounding effects produced by the host's immunologic response to the allograft.

MATERIALS AND METHODS

Animals and Chemicals. Adult male inbred Wistar rats weighing 250–300 g were purchased from Harlan Sprague-Dawley (Indianapolis, Indiana). Diethylstilbestrol, nicotinamide adenine dinucleotide, pyridoxal phosphate, adenosine triphosphate, unlabeled ornithine, sodium molybdate, Tris base, calf thymus DNA, and bovine serum albumin were purchased from Sigma Chemical Company (St. Louis, Missouri). New England Nuclear (Boston, Massachusetts) provided the [^{3}H]estradiol (99 Ci/mmol), [^{14}C]ornithine (57.6 mCi/mmol), 17-alpha-methyl-[^{3}H]methyltrienolone ([^{3}H]R1881) (87 Ci/mmol), and unlabeled R1881. Tritiated thymidine (5 Ci/mmol) and Aqueous Counting Scintillant were obtained from Amersham (Arlington Heights, Illinois). DEAE-cellulose paper was purchased from U.S. Industrial Chemicals Company (Tuscola, Illinois). All other chemicals were obtained from Fisher Scientific Company (Pittsburgh, Pennsylvania).

Surgical Procedures. Adult male rats were randomly allocated to three groups and treated as follows: Group I animals received cyclosporine in olive oil (25 mg/kg orally) for seven days and an intravenous bolus injection of cyclosporine in saline (5 mg/kg) on the eighth day, followed immediately by a standard two-thirds partial hepatectomy (9). Group II animals received the olive oil vehicle orally for seven days and an intravenous injection of saline on the eighth day, which was followed immediately by a standard two-thirds partial hepatectomy. Group III animals received cyclosporine according to the same treatment schedule as the animals in group I; however, they were subjected to a sham operation rather than a two-thirds partial hepatectomy on the eighth day.

All surgical procedures were performed between 9 and 11 AM to eliminate any potential effect of diurnal variations in the parameters assessed. Light ether anesthesia was used for all the surgical procedures and the subsequent sacrifice of the animals studied.

At 6, 24, 48, and 72 hr after partial hepatectomy or sham operation, the rats were anesthetized with ether and weighed. Blood was withdrawn from their abdominal aorta and the remnant liver was removed, weighed, and homogenized in four volumes 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. The homogenate was centrifuged at 103,000*g* for 1 hr at 4° C, and the supernatant obtained was used for all the assays performed.

Estrogen and Androgen Receptors. The specific binding of a saturating concentration of [^{3}H]estradiol was used to determine the cytosolic estrogen receptor activity (10). The hepatic cytosol was diluted 1:1 with a buffer consisting of 40 mM sodium molybdate, 1.5 mM EDTA, and 10 mM Tris HCl (pH 7.4) in order to stabilize the estrogen receptor. A mixture consisting of 200 μl diluted cytosol, 25 μl 30 μM [^{3}H]estradiol and 25 μl ethanol was used to determine total binding of [^{3}H]estradiol. Nonspecific binding was measured in parallel assays in which the ethanol was replaced with 25 μl 3 μM unlabeled DES dissolved in ethanol. In each case, the mixture was allowed to incubate for 2 hr at 4° C and the reaction was terminated by the addition of 0.4 ml 1% dextran-coated charcoal to remove unbound steroid. After centrifuging at 1500*g* for 5 min at 4° C, the supernatant was transferred carefully to a scintillation vial containing 8 ml ACS scintillation fluid. The radioactivity in the supernatant was measured in a Packard Tri-Carb 460 CD liquid scintillation system (Downers Grove, Illinois).

The assay used to quantitate the cytosolic androgen receptor activity was similar to the one described above for the estrogen receptor with minor exceptions (5). Total binding was measured using a mixture of 200 μl of the cytosol with 25 μl tritiated R1881, a synthetic androgen.

Parallel assays in which 25 μl unlabeled R1881 was added to the mixture were used to determine nonspecific binding. The binding of R1881 to glucocorticoid receptors was blocked by adding 5 μm triamcinolone acetonide to the assay mixture. After an overnight incubation at 4° C, the reaction was terminated by adding 0.4 ml 1% dextran-coated charcoal to remove unbound R1881. The mixture was centrifuged at 1500*g* for 5 min at 4° C and the supernatant placed in scintillation vials with 8 ml ACS scintillation fluid. The radioactivity present in the supernatant was counted in a Packard Tri-Carb 460 CD liquid scintillation system.

Thymidine Kinase Activity. The level of thymidine kinase activity was determined by measuring the *in vitro* conversion of thymidine to thymidine phosphate (12). The reaction mixture consisting of 0.1 ml cytosol, 850 μl incubation buffer (5 mM adenosine triphosphate and 3.6 mM magnesium chloride in 50 mM Tris HCl, pH 8.0), and 50 μl 1 μM [^{3}H]thymidine was allowed to incubate for 10 min at 37° C. The reaction was terminated by immersing the assay tubes in boiling water for 2 min. The mixture was centrifuged at 1500*g* for 5 min at 4° C. Aliquots of 0.1 ml each were removed from each assay tube and spotted onto 3.8-cm DEAE-cellulose paper squares. The DEAE-cellulose paper squares were washed in 1 mM ammonium formate for 5 min, distilled water for 3 min, ammonium formate for 5 min, and finally rinsed in distilled water again. The squares were placed in glass scintillation vials and the radioactivity on the paper eluted into solution by the addition of a 0.1 M HCl–0.2 M KCl mixture. After allowing 15 min for elution to occur, 10 ml ACS scintillation fluid was added to each vial. The radioactivity present in solution was counted in a Packard Tri-Carb 460 CD liquid scintillation system.

Miscellaneous Methods. The method of Lowry et al was used to determine the cytosolic protein concentration using bovine serum albumin as the standard (13). Serum measures of liver injury were assessed using a Random Access Chemistry Analyzer (Technicon RA 500).

All results are reported as mean values ± SEM. Statistical analysis of the data was performed using a Student's *t* test. A *P* value of 0.05 or less was considered to represent a significant difference.

RESULTS

The pattern of liver injury observed in the animals studied is shown in Table 1. Note that the measure of liver injury assessed was similar in all groups prior to surgery. A significant increase in AST levels occurred with the hepatic injury of partial hepatectomy (groups I and II). A smaller but nonetheless significant increase in AST levels was present also in the sham-operated animals (group III). Interestingly, the degree of hepatic injury, measured by the serum AST level, was less in the rats pretreated with cyclosporine (group I) than it was in the vehicle-treated animals (group II) following partial hepatectomy.

TABLE 1. SERUM ALANINE AMINOTRANSFERASE (AST*) LEVELS AFTER CYCLOSPORINE TREATMENT AND PARTIAL HEPATECTOMY (GROUP I), VEHICLE TREATMENT AND PARTIAL HEPATECTOMY (GROUP II), AND CYCLOSPORINE TREATMENT AND SHAM OPERATION (GROUP III)

	AST (IU/liter)		
Time (hr)	*Group I*	*Group II*	*Group III*
0	83 ± 18	85 ± 8	83 ± 18
6	1520 ± 407	1146 ± 167	147 ± 15
24	1399 ± 78	2467 ± 663	110 ± 2
48	543 ± 100	1976 ± 800	106 ± 8
72	439 ± 178	NA	75 ± 2

*Alanine aminotransferase.
†Mean ± SEM. *N* = 4–8 animals per time per group.

The effects of pretreatment with cyclosporine and subsequent partial hepatectomy on the cytosolic estrogen receptor activity of liver are shown in Table 2. Preoperative levels of estrogen receptor activity were significantly greater ($P < 0.01$) in the rats treated with cyclosporine (groups I and III) than in the olive oil-treated control group (group II). A significant decrease in estrogen receptor activity was evident 6 hr after operation in all three groups (Table 2). A further decrease in the hepatic estrogen receptor activity occurred between 6 and 72 hr in the cyclosporine-treated animals subjected to the sham operation such that the levels at 72 hr were approximately half those measured preoperatively in the same group. It should be noted, however, that the estrogen receptor activity at this point, in this group of animals (group III), was still greater ($P < 0.05$) than the preoperative level present in the vehicle (olive oil) -treated animals (group II). The cyclosporine-treated rats subjected to partial hepatectomy (group I) experienced a further reduction ($P < 0.01$) in the estrogen receptor activity present in the liver at 24 hr after partial hepatectomy, with the level achieved being approximately one fourth of the preoperative level. Thereafter the estrogen receptor activity increased steadily and returned

TABLE 2. LEVELS OF ESTROGEN RECEPTOR ACTIVITY IN GROUPS I–III*

	Estrogen receptor activity (fmol/mg protein)		
Time (hr)	*Group I*	*Group II*	*Group III*
0	43 ± 4	18 ± 1	43 ± 4
6	30 ± 4	9 ± 0.3	34 ± 0.6
24	10 ± 2	11 ± 2	34 ± 4
48	26 ± 3	14 ± 3	24 ± 1
72	34 ± 5	23 ± 2	23 ± 2

*Mean ± SEM. *N* = 4–8 animals per group per time point.

TABLE 3. LEVELS OF ANDROGEN RECEPTOR ACTIVITY IN GROUPS I–III*

	Androgen receptor activity (fmol/mg protein)		
Time (hr)	*Group I*	*Group II*	*Group III*
0	1.87 ± 0.56	1.99 ± 0.49	1.87 ± 0.56
6	0.62 ± 0.25	0.58 ± 0.19	1.02 ± 0.27
24	2.69 ± 0.60	1.05 ± 0.35	0.53 ± 0.2
48	3.35 ± 0.40	2.0 ± 0.76	0.56 ± 0.28
72	1.42 ± 0.29	NA	1.61 ± 0.15

*Mean ± SEM. N = 4–8 animals per group per time period.

TABLE 4. LEVELS OF ORNITHINE DECARBOXYLASE ACTIVITY IN GROUPS I–III*

	Ornithine decarboxylase activity (cpm/mg protein)		
Time (hr)	*Group I*	*Group II*	*Group III*
0	726 ± 18	546 ± 11	726 ± 18
6	4984 ± 337	3096 ± 785	2573 ± 238
24	3619 ± 631	2068 ± 711	685 ± 18
48	2319 ± 242	2557 ± 128	741 ± 28
72	1322 ± 280	1142 ± 259	664 ± 10

*Mean ± SEM. N = 4–8 animals per group per time period.

almost to the preoperative level by 72 hr following partial hepatectomy. The estrogen receptor response of the vehicle-treated controls subjected to a partial hepatectomy (group II) was qualitatively similar to that of the cyclosporine-treated animals (group I) but occurred at a much reduced initial level of activity and subsequent percent change with time after the partial hepatectomy (Table 2).

The effects of cyclosporine and subsequent partial hepatectomy on the hepatic cytosolic androgen receptor activity are shown in Table 3. The preoperative levels of androgen receptor activity in the cyclosporine-treated and vehicle-treated animals were similar. A reduction in androgen receptor activity occurred at 6 hr after both partial hepatectomy and sham operation with the decline being similar in all three groups (Table 3). The reduction in androgen receptor activity persisted for 48 hr in the sham-operated animals, whereas in both the vehicle-treated partially hepatectomized animals and the cyclosporine-treated partially hepatectomized animals, the receptor activity either increased to (group II) or surpassed (group I) the preoperative level at 48 hr. The hepatic androgen receptor activity at 24 and 48 hr was greater ($P < 0.05$) in rats treated with cyclosporine and partial hepatectomy (group I) than in either of the other two groups.

The changes in ornithine decarboxylase activity observed after partial hepatectomy and sham operation in cyclosporine- and vehicle-treated animals are shown in Table 4. The preoperative levels of ornithine decarboxylase activity were greater in the livers of cyclosporine-treated rats (groups I and III) than those in the vehicle-treated animals (Group II) ($P < 0.01$). A significant increase in the hepatic activity of ornithine decarboxylase activity occurred postoperatively and peaked at 6 hr in all three groups compared to the respective preoperative control levels ($P < 0.01$). The peak level of ornithine decarboxylase activity was greatest in the rats treated with cyclosporine and subjected to a partial hepatectomy (Table 4). Ornithine decarboxylase activity returned toward preoperative levels at 24 hr in all three groups of rats studied but remained elevated ($P < 0.05$) compared to preoperative control levels in both groups subjected to a partial hepatectomy; again the ornithine decarboxylase activity was greatest in the rats treated with cyclosporine (group I).

As was the case with ornithine decarboxylase, the basal preoperative levels of thymidine kinase activity (Table 5) were significantly greater in rats pretreated with cyclosporine (groups I and III) than in the vehicle-treated animals ($P < 0.01$). The thymidine kinase activity in the liver of cyclosporine-treated rats subjected to sham operation (group III) was significantly less at 24 hr than it was preoperatively ($P < 0.05$). Thereafter a delayed and rather minor increase in thymidine kinase activity occurred at 48 and 72 hr after sham operation with the level present at 72 hr being greater than the preoperative basal level ($P < 0.05$) (Table 5). In contrast, a significant increase in thymidine kinase activity was observed at 24 hr after partial hepatectomy (groups I and II), with levels being greater ($P < 0.05$) in the rats pretreated with cyclosporine (group I) as compared to the vehicle treated controls. The increased levels of thymidine kinase activity seen after partial hepatectomy persisted

TABLE 5. LEVELS OF THYMIDINE KINASE ACTIVITY IN GROUPS I–III*

	Thymidine kinase activity (dpm/mg protein)		
Time (hr)	*Group I*	*Group II*	*Group III*
0	4364 ± 847	1346 ± 219	4364 ± 847
6	4403 ± 1242	581 ± 90	1525 ± 101
24	16777 ± 581	11110 ± 1269	1098 ± 76
48	10736 ± 640	12623 ± 1457	3921 ± 749
72	14794 ± 415	11720 ± 459	5422 ± 436

*Mean ± SEM. N = 4–8 animals per group per time period.

throughout the duration of the study with the levels being greater ($P < 0.05$) always in the rats pretreated with cyclosporine (group I) as compared to the animals receiving the olive oil vehicle.

DISCUSSION

The important results of this study are that the preoperative levels of estrogen receptors present in the liver of animals treated with cyclosporine are greater than they are in rats receiving the olive oil vehicle. In contrast to the changes observed for the activity of estrogen receptors, the androgen receptor activity in hepatic cytosol was not affected by cyclosporine treatment. The changes in both the estrogen and the androgen receptor activity content of the liver after partial hepatectomy in the vehicle-treated animals were similar to those described previously for normal animals (1, 5, 14). Similar qualitative changes were seen after partial hepatectomy in the cyclosporine-treated rats. However, the quantitative changes were greater for both the estrogen and the androgen receptor. Moreover the potential for a regenerative response to a hepatic injury determined by measuring ornithine decarboxylase activity and thymidine kinase activity was greater in the animals treated with cyclosporine (group I) than it was in the vehicle-treated controls (group II).

Certain liver functions have been noted to display sexual dimorphism in mammals (2–4). Some of these functions may be critical for a completely successful outcome following liver transplantation. Because cyclosporine is used routinely after liver transplantation and because it is known to affect hair growth and Leydig cell function, and the sex hormone status of the liver is modified in response to or as part of the regenerative response, it would appear to be important to investigate the effect of cyclosporine on the sex hormone receptor status of the liver both before and after a surgical procedure associated with a hepatic regenerative response. Thus, in this study, both the estrogen receptor and androgen receptor activity in the hepatic cytosol was studied in response to prior cyclosporine treatment and to a regenerative stimulus, a two-thirds partial hepatectomy. It is of considerable interest that the estrogen receptor but not the androgen receptor activity of the liver is modified by cyclosporine therapy. Although no qualitative differences in the estrogen receptor and androgen receptor changes associated with hepatic regeneration occurring after a two-thirds partial hepatectomy were observed, significant quantitative changes in these two functions in the liver between treatment groups was evident. These changes are particularly interesting when the measures of hepatic injury determined after partial hepatectomy in the two groups of animals subjected to this operative injury are examined. Specifically the injury in the cyclosporine-treated animals was significantly less than that seen in the vehicle-treated controls.

During liver transplantation, the graft liver can be injured in a number of different ways, including an ischemic injury related to procurement and preservation, an immunological injury as a result of the attack mounted by the recipient's immune response to the graft, an infectious attack, and finally, injury occurring as a result of the toxicity of certain drugs, particularly cyclosporine, used following transplantation. Occasionally, because of a mismatch between the size of the donor and the recipient, a small liver is transplanted into a larger recipient and hepatic regeneration of the liver graft is required to meet the metabolic needs of the recipient (15, 16). Similarly, because of the lack of suitable pediatric donors, some centers use partially hepatectomized adult livers in pediatric recipients (17). Both of these situations necessitate a regenerative response in the transplanted liver (15, 16). Moreover, a normal regenerative response in these particular situations may be crucial for the ultimate success of the liver transplant procedure.

Previous studies have shown a temporal relationship between the transfer of cytosolic estrogen receptor to the nuclear compartment and the onset of DNA synthesis in regenerating liver (14, 18). These observations have implicated estrogens and their receptors as playing an important role in the process of hepatic regeneration. Relevant to these findings are the results reported herein, which demonstrate a greater level of estrogen receptor activity in the hepatic cytosol of rats treated with cyclosporine.

Estrogens have been implicated as playing a crucial role in the pathogenesis of a number of pathological states of the liver (19–24). Of particular interest in this regard is the role that estrogens and their receptors appear to play in the pathogenesis of hepatic neoplasia (19, 20). Livers containing hepatic adenoma and focal nodular hyperplasia have been shown to have an increased estrogen receptor content (25). The data presented here demonstrate that the livers of cyclosporine-treated rats also have an

increased estrogen receptor activity. This finding may have important implications. As the number of liver transplants performed increases and as survival rates improve, the number of long-term survivors after liver transplantation will increase. The possible effect of an increased estrogen receptor activity in the livers of these cyclosporine-treated survivors is currently unknown.

It is of some interest that previous studies have shown that prolactin plays an important role in the regulation of the cytosolic content of estrogen receptors in liver (27). Specifically, the decrease in hepatic cytosolic estrogen receptor content seen after hypophysectomy can be restored partially by the administration of exogenous prolactin (26). Recently it has been shown that the administration of cyclosporine to rats causes an increase in their prolactin levels (27). Thus, the increase in prolactin levels associated with cyclosporine may be responsible, at least in part, for the enhanced activity of the estrogen receptor observed in this study.

Regardless of the specific mechanism involved, the results of the present studies demonstrate that treatment with cyclosporine results in an increase in hepatic content of estrogen receptor activity within the liver and that this is associated with an increase in activity within the liver of thymidine kinase and orthinithine decarboxylase. It is interesting to speculate also that the changes observed in the hepatic content of estrogen and androgen receptor levels in the cyclosporine-treated animals studied, if also present in other tissues, may explain, at least in part, some of the untoward consequences of cyclosporine treatment observed clinically.

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