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Liver Pathology and Cell Proliferation After Calcineurin Inhibitors and Antiproliferative Drugs Following Partial Hepatectomy in Rats

H. Kirimlioglu, V. Kirimlioglu, S. Yilmaz, S. Coban, E. Turkmen, and C. Ara

ABSTRACT

Immunosuppressants are the cornerstones of treatment after solid organ transplantation. This study investigated the pathology and cell proliferation following partial hepatectomy (PH) in rats undergoing immunosuppressive treatment. After 1 day, all rats were subjected to 70% PH. Groups A and B (n = 10) received calcineurin inhibitors subcutaneously: either FK506 or cyclosporine (CyA). Groups C and D (n = 10) received antiproliferative drugs: either mycophenolate mofetil (MMF) or sirolimus (SRL) by gavage. A control group (n = 5) received 1 mL of tap water daily. On postoperative day 2, all rats were sacrificed to obtain liver tissue for pathologic examination. Using immunohistochemistry we separately examined the hepatectomy surface and the liver parenchyma. In the parenchyma, the Ki-67 indices were higher in the CyA and FK506 groups and lower in the SRL and MMF groups compared with controls ($P < .01$). CyA had the highest and MMF the lowest values. On the hepatectomy surface, Ki-67 indices and TGF-alpha expressions were higher in the CyA group and lower in the SRL and MMF groups compared with the control group ($P < .01$). Slightly higher values in the FK506 group were not significantly different compared with the control group ($P > .05$). All groups other than FK506 showed prominent cholangiolar epithelial phenotypes compared with the control group. In the CyA and SRL groups, the number of cholangiolar cells was higher ($P < .01$), and in the MMF group lower than in the control group ($P < .01$). Among all groups, SRL had the highest values.

IMMUNOSUPPRESSANTS are cornerstones of treatment following solid organ transplantation. Today split liver grafts are no longer reserved for children but are employed with increasing frequency in adults. The liver shows a high capacity for regeneration after partial hepatectomy (PH).[1,2] Following PH, cell proliferation does not ensue at the level of the cut; new lobes do not develop to take the place of those removed.[2] There is hyperplasia of the remaining lobes which reaches 50% at 16 to 24 hours after 70.6% PH in the rat.[2] Over the last years this topic has been the subject of extensive animal research which suggests the roles of a variety of factors: Follistatin,[3] interferon gamma,[4] and immunosuppressants.[1,5] Our previous study showed that the onset of liver regeneration after PH is inhibited by the use of new immunosuppressive drugs.[1]

MATERIALS AND METHODS

Male Swiss albino rats weighing about 200 to 250 g were obtained from Fırat University, Animal Laboratory, Elazig, Turkey. All experiments were performed in accordance with the guidelines for Animal Research from the National Institutes of Health and were approved by our Committee on Animal Research. The animals were housed in stainless-steel cages under controlled temperature and humidity conditions and in a quiet room with a 12/12-hour light/dark cycle. Rats were maintained on a standard laboratory diet with tap water ad libitum throughout the experiment, except for an overnight fast before surgery. All surgical procedures were performed under sterile conditions. The animals underwent PH according to the method of Higgins and Anderson,[2] while sedated with intraperitoneal ketamine (50 mg/kg) and xylazine HCl (10 mg/kg) anesthesia. Briefly, for a 70% hepatectomy the median lobe, left lateral lobe, and right lateral lobe were resected. All animals were treated with 100 mg/kg mezlocillin (Baypen, Bayer, Istanbul, Turkey) by intramuscular injection once at the time of surgery.

From the Departments of Pathology (H.K., E.T.) and General Surgery (V.K., S.Y., S.C., C.A.), Inonu University, School of Medicine, Malatya, Turkey.

Address reprint requests to Vedat Kirimlioglu, MD, Department of General Surgery, Inonu University, Turgut Ozal Medical Center, Malatya, Turkey. E-mail: vkirimlioglu@inonu.edu.tr

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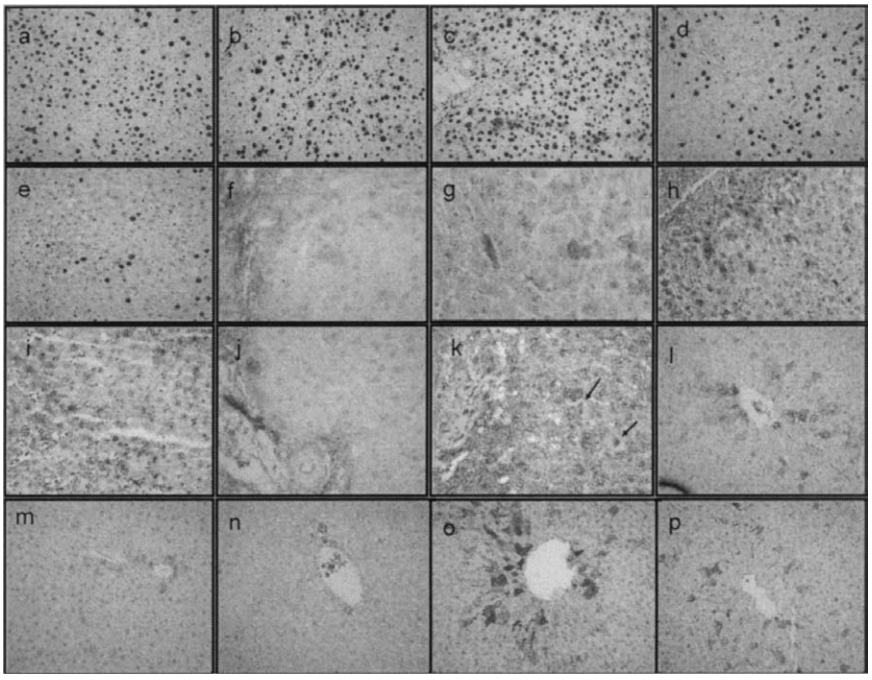

Fig 1. When the Ki-67-labeling indices were compared with the control **(a),** FK506 had the higher **(b),** CyA the highest **(c),** SRL the lower **(d),** and MMF the lowest **(e)** indices. Cytokeratin E1 was used to determine the cholangiolar phenotype in the hepatectomy surface. When compared with the control group **(f),** in the FK506 **(g)** and CyA **(h)** groups, the cells were increased and decreased in the MMF **(j)** group similar to the hepatocytes, though the SRL **(i)** group had the greatest increase. Hepatocyte antibody had a correlated staining pattern with cytokeratin E1; in the FK506 **(k)** group, hepatocyte antibody was negative in some of the cells (arrows) at the hepatectomy surface. TGF-alpha indices were correlated with Ki-67 indices. The control **(l),** SRL **(m),** MMF **(n),** CyA **(o),** and FK506 **(p)** groups were seen.

After the operation, the animals were given a single 5 mL subcutaneous injection of 10% glucose solution (Biosel, Turkey) and received ad libitum 20% glucose solution (Biosel, Turkey) in tap water accompanied by normal rat chow. Animals received various immunossuppressive drugs at standard doses 24 hours before and immediately (0 hours), 24, and 48 hours after PH. Groups A and B (n = 10) received calcineurin inhibitors subcutaneously: group A, cyclosporine (CyA; 5 mg/kg/d; Sandimmun, Novartis) and group B, FK506 (1 mg/kg/d; Prograf, Eczacibasi). Groups C and D (n = 10) were treated with antiproliferative drugs by gavage: group C, mycophenolate mofetil (MMF; 40 mg/kg/d; Cellcept, Roche) and group D, sirolimus (SRL; 2.5 mg/kg/d; Rapamune, Wyeth). A control group (n = 5) received 1 mL of tap water daily. On postoperative day 2, animals were sacrificed by exsanguination.

Pathologic Analysis

Wedge liver biopsies were taken from each animal for histological examination. The hepatectomy surface and liver parencyhma were examined separately. Liver tissues were fixed in 10% neutral formalin, embedded in paraffin, and examined in 4-μm-thick sections. Histochemical examination with hematoxylin-eosin and Masson's trichrome stains was performed to evaluate lobular architecture, inflammatory infiltrates, and fibroblast content. Cellular proliferation was detected with immunohistochemistry based on the streptavidin-biotin peroxidase method (Lab Vision, Calif, USA). Sections (4 μm thick) were dewaxed in xylene and hydrated through graded concentrations of alcohol. Endogenous peroxidase activity was blocked with 1% hydrogen peroxidase for 10 minutes. Sections from hepatectomy were stained with antibodies to cytokeratin E1 (Lab Vision, Calif, USA), and hepatocyte Ab-1 (Lab Vision, Calif, USA) to detect regenerating cells of biliary versus hepatocyte phenotype. Both the sections from the hepatectomy surface and the liver parenchyma were stained with Ki-67 (Lab Vision, Calif, USA) to evaluate the degree of cell proliferation and TGF-alpha as indices of the effect of immunosuppressive drugs on liver regeneration. At the hepatectomy surface, 1000 hepatocytes were counted; the number of cells positively stained with CK E1 were noted for each drug and for the control group.

The Ki-67 and TGF-alpha indices were determined in both the sections from the hepatectomy surface and from the liver parenchyma. In these sections we counted 1000 hepatocytes; the numbers of Ki-67 and TGF-alpha immunopositive cells in the area were considered the indices. This procedure was repeated five times for each marker to achieve statistical analysis among the groups.

Statistical Analysis

The results are expressed as mean values ± SD. For statistical purposes, a Mann-Whitney nonparametric test was employed with $P < .05$ considered significant.

RESULTS

Pathology of Liver Biopsies

Sections from the hepatectomy surface in all groups showed changes of liver regeneration. This process was mediated by proliferation of viable hepatocytes that formed trabeculae or were characterized by eosinophilic cytoplasm. The proliferating hepatocytes displayed either an hepatocyte (hepatocyte Ab-1) or a cholangiolar epithelial phenotype (cytokeratin E1, CK E1) (Fig 1). There was also polymorphonuclear infiltration and fibroblastic proliferation around the ischemic areas. Sections from the liver parenchyma and the hepatectomy surface showed Kupffer cell hypertrophy.

Statistical Analysis

In the parenchyma, the Ki-67 indices were higher for the CyA and FK506 groups, and lower for the SRL and MMF groups compared with controls ($P < .01$). CyA showed the

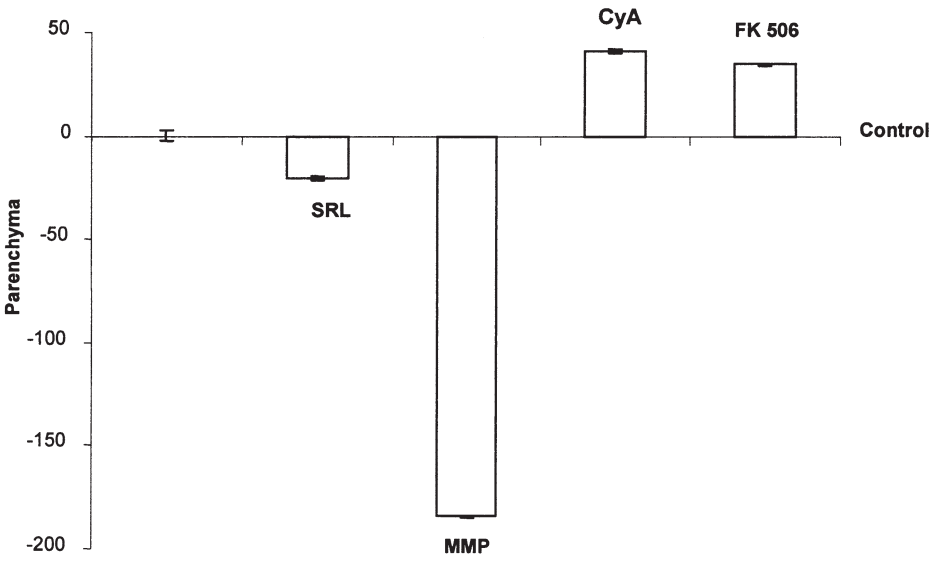

Fig 2. In the parenchyma, the comparison of the Ki-67 indices of the immunosuppressive drugs with the control group.

highest and MMF the lowest Ki-67 values (Fig 2). On the hepatectomy surface, Ki-67 indices were higher in the CyA group and lower in the SRL and MMF groups ($P < .01$), but the slightly higher values in the FK506 group did not show a significant difference compared with the control group ($P > .01$) (Fig 3). Ki-67 indices and TGF-alpha indices were similar (Fig 4).

All groups other than FK506 showed prominent differences in cells displaying cholangiolar epithelial phenotype compared with the control group (Fig 5). In the CyA and SRL groups, the number of cholangiolar cells was higher ($P < .01$) and in the MMF group, lower than the control group ($P < .01$). Among all groups, SRL showed the highest values (Fig 5).

DISCUSSION

This study shows that SRL and MMF inhibit and calcineurin inhibitors augment liver regeneration after an experimental 70% PH in rats. These results agree with previous studies.[1,6–10] To our knowledge, there is no study in the literature conducted on partially hepatectomized rats that investigated the histopathological findings of hepatocytes and cholangioles.

SRL is a promising new immunosuppressive agent with a unique mechanism of action to disrupt costimulatory and cytokine stimulated T-cell activation through inhibition of a multifunctional P 70 S 6 kinase and 4E-BPI phosphorylation.[11,12] SRL, when bound to its intracellular receptor

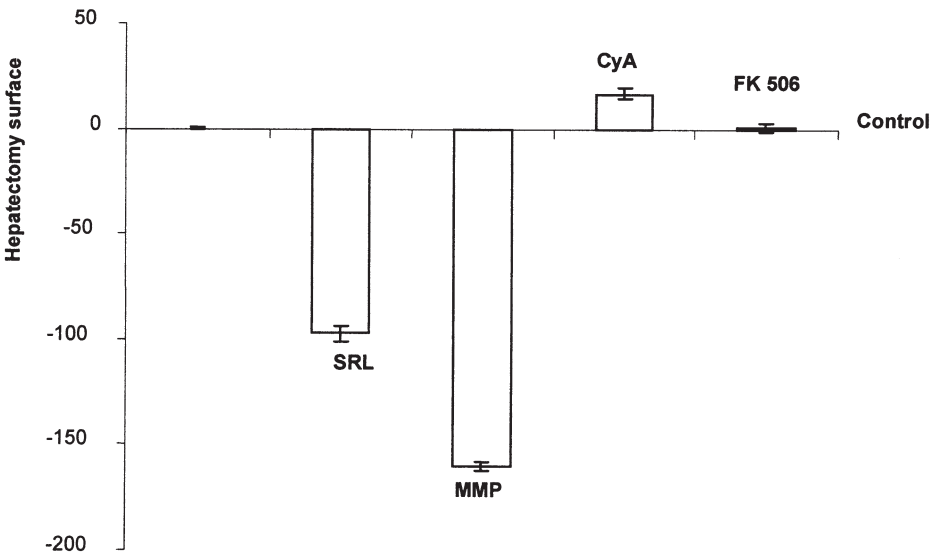

Fig 3. In the hepatectomy surface, the comparison of the Ki-67 indices of the immunosuppressive drugs with the control group.

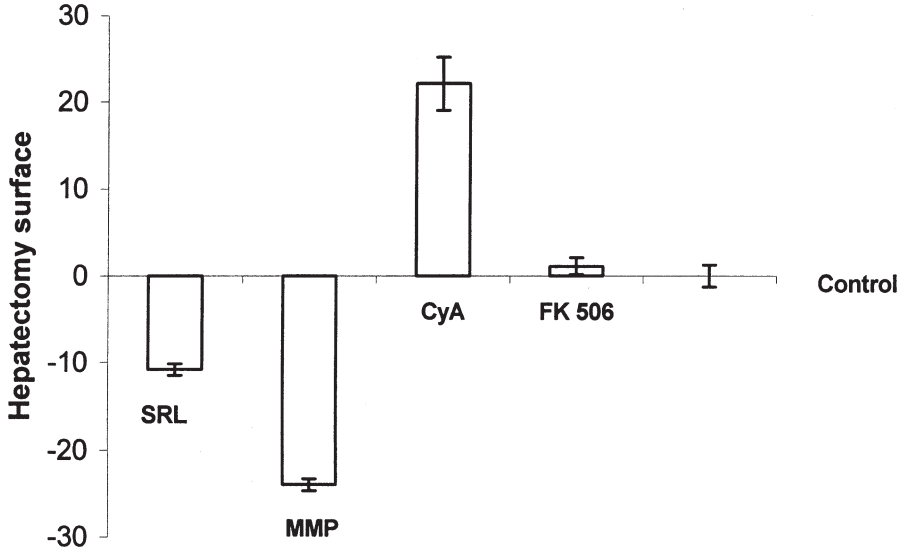

Fig 4. In the hepatectomy surface, the comparison of the TGF-alpha indices of the immunosuppressive drugs with the control group.

FKBP12, inhibits the function of the target of rapamycin (mTOR), a protein kinase whose catalytic domain is structurally related to that of phosphatidyl s-kinase.[13,14] The mechanism that impairs the regeneration of the liver after PH is that SRL selectively inhibits P 70 S 6 kinase activation, but not the functional phosphorylation of 4E-BP.[12] On the other hand, MMF is a selective and reversible uncompetitive inhibitor of inosine monophosphate dehydrogenase (IMPDH) that is crucial for proliferation of B and T lymphocytes. Selective inhibition of guanine reduces DNA synthesis of a variety of immunologic and other specialized cells, including hepatocytes.[6,11]

Calcineurin inhibitors promote liver regeneration by a nonimmunological pathway.[8,15] Treatment with CyA and FK506, which inhibit IL-2 production, increased the mitotic indices of regenerating liver.[16] It has been hypothesized that NK cells in the hepatic sinusoids control liver regeneration, because the NK cells exhibit cytotoxicity against regenerating hepatocytes in the 70% PH model.[16,17] Tamura et al's[16] observations suggest that FK506 promotes liver regeneration, which is attributable to inhibition of the number and activity of liver resident NK cells rather than to changes in hepatic growth factor (HGF) or transforming growth factor beta (TGF-beta). Twenty-four hours after PH, Kahn et al[15] observed a significant increase in cytosolic ornithine decarboxylase and thymidine kinase activity compared with the vehicle-treated animals.

In conclusion, the results of this study showed that the proliferative effects of CyA are greater than those of FK506. Between the antiproliferative drugs, MMF was the most potent suppressor of liver regeneration. Besides its antiproliferative effect among the four drugs, SRL enhanced cholangiolar epithelial cells to the greatest degree. These findings suggest the need for further studies to assess

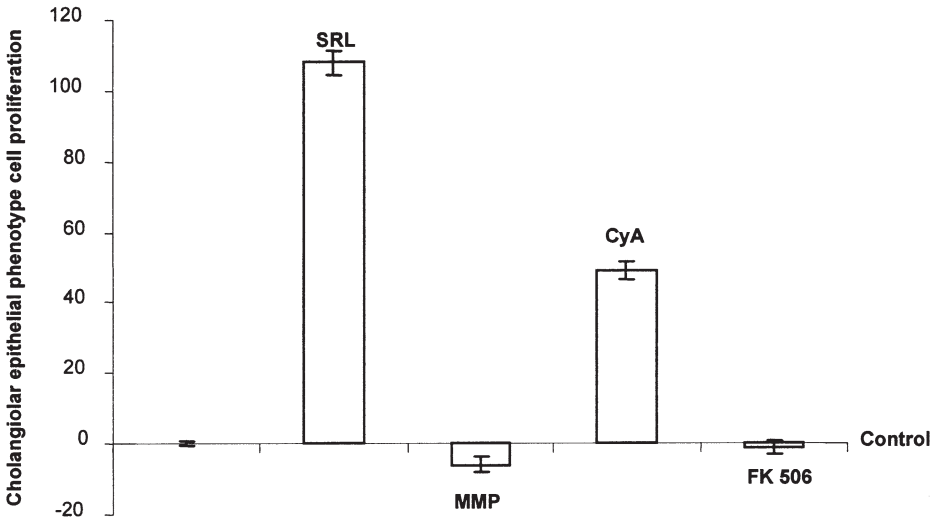

Fig 5. In the cholangiolar epithelial phenotype, the comparison of the Ki-67 indices of the immunosuppressive drugs with the control group.

the use of SRL to prevent bile duct vanishing syndrome in chronic rejection.

The effects of calcineurin inhibitors and antiproliferative drugs on TGF-alpha are believed to be on hepatocytes rather than cells of the cholangiolar epithelial phenotype. Further it remains unclear whether stimulation or rather inhibition of hepatocyte and cholangiolar proliferation would be advantageous. Forcing hepatocytes to divide might compromise their limited number to fulfill the metabolic demands, possibly leading to the death of the animal. In contrast, inhibition of regeneration may increase the metabolic performance of the remaining hepatocytes and cholangioles leading to the survival of the animal, or in contrast, to death due to an insufficient number of metabolically active cells. Based on these results, partial liver graft recipients should be treated with calcineurin inhibitors rather than antiproliferative drugs, particularly MMF. However, the use of SRL may be justified in tumor cases.

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