Hepatic Growth Effects of Methylprednisolone, Azathioprine, Mycophenolic Acid, and Mizoribine

Cyclosporine (CSA) and FK506 augment liver regeneration after partial hepatectomy in rats (1, 2), an effect that is not the consequence of immune modulation (3). In addition, both drugs increase the hepatocyte proliferative response in dogs that is characteristic after Eck fistula, and they also prevent the hepatocyte atrophy and organelle disruption that is caused by this operation (4). Substances changes these hepatocytes have been termed hepatoatrophy. Rapamycin (RPM) (5), a third potent immunosuppressive has opposite (antihepatotropic) actions without evidence of inherent hepatocyte toxicity (5).

A hypothesis that these growth control alterations are caused by altered signal transduction of the hepatocytes independent of immunologic pathways (5) has been supported in vivo and in vitro experimentation (3). However, there is a paucity of information about the influence of other immunosuppressive drugs on growth and regeneration. Using in vivo (partial hepatectomy) and in vitro (tissue culture) rat models described elsewhere (2, 3, 5, 6), we have investigated the effect upon hepatocyte proliferation of methylprednisolone (MP), mycophenolic acid (MPA), mizoribine (MZ), and azathioprine (AZA).

In vivo experiments were performed with adult male inbred Fisher 344 rats weighing 180–200 g (Hilltop Lab Animals, Scottsdale, PA). The rats were given standard rat laboratory diet and water ad libitum, and kept in a temperature and light–control room (light 0730–1930). Immunosuppressive drugs were given preoperatively on days 1, 2, 3, 5, and 10 (Table 1) that were sacrificed at 36 hr. The liver specimens from these animals were formalin-fixed, stained with H&E, and examined for the percentage of mitoses.

The in vivo experimental groups are summarized in Table 1. After partial hepatectomy, the contemporaneous vehicle controls showed considerable variability from group to group (Fig. 1). In control experiments, the highest DNA synthesis and mitotic rates were in control animals injected with saline (Table 1, groups 1 and 2) whereas the lowest were in animals with drug vehicle containing CMC solvent alone (group 9) and CMC mixed with Tween and alcohol (group 5). This suggested a potential inhibitory effect of the CMC carrier and emphasized the need for concurrent controls for each test group.

The only drug that significantly augmented regeneration relative to the controls was MP, P<0.01 (Fig. 1). The only drug that significantly inhibited regeneration was MZ (P<0.01). The MZ inhibition was profound, reducing DNA synthesis >85% and the mitosis rate >70% (Fig. 1). MPA at a standard dose and AZA at low (1 mg/kg oral) and high (6 mg/kg i.m.) doses were without effect on regeneration in the whole animal (Fig. 1).

In vitro experiments were performed with hepatocyte culture preparations made from the livers of the same strain of unaltered rats as used in vivo. Mitotic indices were obtained in 3 animals in the groups 1, 3, 5, 7 and 9, and in 6 animals in the groups 2, 4, 6, 8 and 10 (Table 1) that were sacrificed at 33 hr. The liver specimens from these animals were formalin-fixed, stained with H&E, and examined for the percentage of mitoses.

The in vivo experimental groups are summarized in Table 1. After partial hepatectomy, the contemporaneous vehicle controls showed considerable variability from group to group (Fig. 1). In control experiments, the highest DNA synthesis and mitotic rates were in control animals injected with saline (Table 1, groups 1 and 2) whereas the lowest were in animals with drug vehicle containing CMC solvent alone (group 9) and CMC mixed with Tween and alcohol (group 5). This suggested a potential inhibitory effect of the CMC carrier and emphasized the need for concurrent controls for each test group.

The only drug that significantly augmented regeneration relative to the controls was MP, P<0.01 (Fig. 1). The only drug that significantly inhibited regeneration was MZ (P<0.01). The MZ inhibition was profound, reducing DNA synthesis >85% and the mitosis rate >70% (Fig. 1). MPA at a standard dose and AZA at low (1 mg/kg oral) and high (6 mg/kg i.m.) doses were without effect on regeneration in the whole animal (Fig. 1).

In vitro experiments were performed with hepatocyte culture preparations made from the livers of the same strain of unaltered rats as used in vivo. Mitotic indices were obtained in 3 animals in the groups 1, 3, 5, 7 and 9, and in 6 animals in the groups 2, 4, 6, 8 and 10 (Table 1) that were sacrificed at 33 hr. The liver specimens from these animals were formalin-fixed, stained with H&E, and examined for the percentage of mitoses.

The in vivo experimental groups are summarized in Table 1. After partial hepatectomy, the contemporaneous vehicle controls showed considerable variability from group to group (Fig. 1). In control experiments, the highest DNA synthesis and mitotic rates were in control animals injected with saline (Table 1, groups 1 and 2) whereas the lowest were in animals with drug vehicle containing CMC solvent alone (group 9) and CMC mixed with Tween and alcohol (group 5). This suggested a potential inhibitory effect of the CMC carrier and emphasized the need for concurrent controls for each test group.

The only drug that significantly augmented regeneration relative to the controls was MP, P<0.01 (Fig. 1). The only drug that significantly inhibited regeneration was MZ (P<0.01). The MZ inhibition was profound, reducing DNA synthesis >85% and the mitosis rate >70% (Fig. 1). MPA at a standard dose and AZA at low (1 mg/kg oral) and high (6 mg/kg i.m.) doses were without effect on regeneration in the whole animal (Fig. 1).

In vitro experiments were performed with hepatocyte culture preparations made from the livers of the same strain of unaltered rats as used in vivo. Mitotic indices were obtained in 3 animals in the groups 1, 3, 5, 7 and 9, and in 6 animals in the groups 2, 4, 6, 8 and 10 (Table 1) that were sacrificed at 33 hr. The liver specimens from these animals were formalin-fixed, stained with H&E, and examined for the percentage of mitoses.
tered male rats (180 and 200 g). Hepatocytes were isolated with a previously standardized in situ two-step collagenase perfusion technique (3, 6). The hepatocytes were dispersed and washed twice with cold Ca2+-free perfusion buffer and resuspended in basal medium (MEM) supplemented with pyruvate (1 mM), proline (0.26 mM), insulin (10-7 M), and 5% FCS. Cell number was determined with a hepatocytometer. Viability was determined by trypan blue exclusion. For drug testing, only preparations with >95% viability were used. DNA content of the harvest cells was determined by a microfluorometric method (3, 6). The hepatocytes were plated at a cell density of 2 x 105/4 well in a Corning 35-mm tissue culture dish containing 1.5 ml medium and maintained at 37°C in a 5% CO2 atmosphere. Preliminary viability studies in which the basal medium was enriched with insulin but not with EGF, the viability of these resting hepatocytes over the 48-hr following a 3-hr attachment was remarkably different than those reported by Kim et al. (9) who observed striking augmentation of DNA synthesis in essentially the same experiments. They interpreted this as evidence that hepatic regeneration is immune-modulated. Be-

TABLE 2. Viability, DNA concentration, and transaminase levels of hepatocytes in media enriched with immunosuppressive drugs in the absence of EGF.

<table>
<thead>
<tr>
<th></th>
<th>Viability percentage (%)</th>
<th>DNA concentration (μg/dish)</th>
<th>ALT (U/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>95±4</td>
<td>3.2±0.2</td>
<td>5.1±1</td>
</tr>
<tr>
<td>MPA 10 μg/ml</td>
<td>94±3</td>
<td>3.2±0.3</td>
<td>4.2±0.6</td>
</tr>
<tr>
<td>AZA 1 μg/ml</td>
<td>56±4</td>
<td>3.1±0.3</td>
<td>16±1</td>
</tr>
<tr>
<td>MZ 100 ng/ml</td>
<td>95±6</td>
<td>3.3±0.2</td>
<td>4.5±0.7</td>
</tr>
<tr>
<td>MP 10 μg/ml</td>
<td>94±3</td>
<td>3.3±0.3</td>
<td>5.1±0.8</td>
</tr>
</tbody>
</table>

* MPA: mycophenolic acid; AZA: azathioprine; MZ: mizoribine; MP: methylprednisolone.

**Figure 1.** 3H-thymidine incorporation and mitoses/1000 hepatocytes in 70% hepatoprotected rats treated or not treated with methylprednisolone (MP), mycophenolic acid (MPA), mizoribine (MZ), and azathioprine (AZA). Drug doses are as shown in Table 1. Twenty-one animals were used for each drug; 8 rats were used as control and 13 were treated with the drugs. Three rats of each control group and six of each experimental group were used for determinations of the number of mitoses. Bars are mean ± SD. (*)P<0.01 (drug versus control).

**Figure 2.** Doses versus DNA synthesis response with MP, MPA, and MZ of cultured hepatocytes (n=9 for all data points (3 experiments with triplicate determinations). Mean ± SD; (*)P<0.05; (**)P<0.01.
cause of the possibility that Kim (9) might have interposed a delay between the last drug dose and the partial hepatectomy, thereby allowing the beginning of regeneration in response to a chemical injury, we performed more experiments in which the last azathioprine dose was not given at the time of operation. Although the animals with the premature cessation of AZA treatment tended to have more vigorous regeneration (Table 3), this difference was not statistically significant. However, the possibility of a "timing" artefact has not been ruled out and will require further study.

Unlike AZA and the other cytotoxic drugs (MPA and MZ), MP caused a striking increase of proliferation of hepatocytes in culture and it augmented liver regeneration in the intact animal. Such steroid-stimulating effects on hepatocyte division have not been noted before—and, in fact, Richman et al. (10) reported inhibition by dexamethasone, which in our model also was stimulatory (unpublished observations). MP is known to inhibit the synthesis and expression of multiple cytokines including IL1, IL6, and migration inhibitor factor (11, 12). Although IL1 and IL6 are thought from reported in vitro experiments to be growth suppressors (13) this could not be demonstrated by us in a sensitive in vivo test system in which the recombinant cytokines in question were infused directly into the tied-off portal vein of the ECK fistula liver (14). Thus, the proliferative response to MP reported herein both in vivo and in vitro cannot be explained by what is currently known about steroid actions.

In contrast, the inhibition of hepatocyte proliferation by cytotoxic drugs is consistent with previous reports about AZA (15). A seemingly obvious explanation could be that AZA, MPA, and MZ selectively inhibit synthesis of purine nucleotides (adenine with AZA and guanine with MPA and MZ) that are required for DNA synthesis.

The molecular basis of growth alterations by immunosuppressants is best understood with FK506, CsA, and RPM—drugs that alter signal transduction with pleiotropic effects not limited to immune cells (3, 8). These agents, which are not inherently cytotoxic, are inactive "prodrugs" until they bind with ubiquitous cytosolic immunophilins and form complexes that act upon a common target(s) (8). Although these molecular mechanisms have been determined principally with the lymphocyte and mast cell, the hepatocyte may be the best to investigate growth control (6).

As the search continues for immunosuppressive drugs, it is self-evident that growth control qualities should be systematically determined. Conversely, drugs under study primarily for their hepatocyte growth control activity (14) should also be screened for possible immunosuppressive activity.

Table 3. Effects on hepatocyte proliferation of AZA administration

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Operation</th>
<th>AZA administration</th>
<th>Route</th>
<th>cpm/mg DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5</td>
<td>Sham</td>
<td>1.5 mg/kg for 3 days</td>
<td>PO</td>
<td>4.477±4.420</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>70% PH</td>
<td>1.5 mg/kg for 3 days</td>
<td>PO</td>
<td>87.574±18.315</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>70% PH</td>
<td>1.5 mg/kg for 4 days</td>
<td>PO</td>
<td>55.318±15.422</td>
</tr>
</tbody>
</table>

Groups 1 and 2 were treated for 3 days but did not receive AZA on the day of the operation as in our experiments shown in Figure 1 (group 3 in this table).

REFERENCES

Received 19 December 1991.
Accepted 11 September 1992.