Regulation of Hepatic Regeneration in Rats by Synergistic Action of Insulin and Glucagon

(growth regulation/portal blood hepatotrophic factors/evisceration/pancreatectomy)

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ABSTRACT Rats were subjected to resection of the gastrointestinal tract, pancreas, and spleen, and maintained by continuous intravenous infusion. Such animals, receiving only electrolytes and glucose, and deprived of a portal blood supply, responded to 68% hepatectomy with a significant rise in hepatic DNA synthesis, which was, however, greatly delayed and diminished compared to normal controls. The activity was restored to normal by infusion of insulin and glucagon (supplemented with glucose and amino acids), but not by either hormone alone; it was not decreased by delaying the start of hormone treatment for 6-7 hr after partial hepatectomy. Infusion of insulin and glucagon into a small series of rats with intact livers did not appreciably elevate DNA synthesis. In normal rats partial hepatectomy is followed by an abrupt fall in portal vein insulin concentration. These findings are all consistent with the suggestion that agents other than insulin and glucagon may serve to initiate hepatic regeneration, but that these two hormones acting synergistically are major regulators of the rate and perhaps also the extent of the regenerative process.

Although mitosis in the liver of the adult rat is a rare event, liver cells proliferate actively in response to partial hepatectomy. This growth process is under humoral control (1), and a variety of agents including hormones, polypeptides, and metabolites have been proposed as potential regulators.

Increasingly, evidence has singled out portal venous blood as a likely source of such agents (2-11). Much of this evidence, though persuasive, is circumstantial, resting upon intricate surgical manipulations of the blood supply to the liver or to liver transplants; livers deprived of portal blood undergo partial atrophy; Staral and his coworkers (2), on the basis of elaborate vascular transpositions in dogs, coupled with DNA labeling, and cyclic nucleotide and enzyme assays, implicated insulin or insulin plus glucagon. Orloff and his associates (3, 10), exploring effects of ablation or transplantation of various splanchnic viscera upon liver regeneration in rats, also designated the pancreatic hormones. Fisher et al. (8), however, on the basis of somewhat similar studies, considered the most likely origin of hepatotrophic factors to be the small intestine, especially the terminal part of the ileum.

An approach permitting more direct evaluation of the role of portal blood factors is provided by portal splanchnic evisceration. Price and his coworkers studied hepatic regeneration in dogs, maintained by continuous intravenous alimentation, following resection of the gastrointestinal tract, pancreas, and spleen, with shunting of blood from the aorta or vena cava into the portal vein; partial hepatectomy induced a modest regenerative response, as evidenced by incorporation of labeled thymidine into hepatocyte DNA, and by mitotic activity (4, 5). We have made similar observations in rats, showing, in addition, that no portal blood flow is required (12). Although the experimental conditions differed in several other respects also, both dogs and rats received insulin. We report here our further use of the eviscerated rat model to explore the function of the pancreatic hormones in hepatic regeneration; the findings assign a major regulatory role to the synergistic action of insulin and glucagon, but suggest a likely involvement of other agents as well.

MATERIALS AND METHODS

Crystalline porcine glucagon and crystalline porcine zinc insulin, donated by the Lilly Research Laboratories, Indianapolis, Ind., were substituted for commercial hormone preparations where indicated.

The experimental procedure previously reported (12) was somewhat modified. Male Sprague-Dawley rats (Holtzman Co., Madison, Wisc.) weighing about 200 g were anesthetized with ether. Using clean but not aseptic technique (instruments, infusion assembly, and solutions were sterile), we placed an indwelling silastic cannula in the right external jugular vein. The cannula consisted of 2 cm of medical grade silastic tubing (0.020 inches (0.51 mm) inside diameter × 0.037 inches (0.94 mm) outside diameter, Dow Corning, Midland, Mich.) connected to a short length of polyethylene tubing (Clay Adams PE 50). The jugular cannula emerged through the skin at the back of the neck, where it was anchored with a suture and adhesive tape. It was coupled to Teflon tubing leading via a swivel to a motor-driven syringe. Infusion of a basic mixture of electrolytes (Table 1) was begun at a rate of 17 ml/24 hr. The previously reported procedure was followed in canulating the bile duct with polyethylene tubing (PE 10) to afford external drainage, and in resecting the gastrointestinal tract, pancreas, and spleen with preservation of the hepatic artery, the portal vein having been ligated and sectioned. Thus all portal blood flow was eliminated (12). The cut ends of the esophagus and rectum were cauterized with heat (a small soldering iron). Resection of the main lobes of the liver (68% hepatectomy) was carried out at this point. The infusion formula was then changed to one containing the requisite hormones and nutrients, and the infusion was continued for the duration of the experiment. We rinsed the abdominal cavity with 0.5% neomycin sulfate before closure, performed a cervical esophagostomy for salivary drainage, injected 5 ml of 5% glucose subeutaneously to combat acute postoperative hypoglycemia (12-14), and terminated the anesthesia. The surgical procedure could usually be completed

Abbreviation: dpm, disintegrations per minute.
TABLE 1. Composition of infusion mixtures

<table>
<thead>
<tr>
<th>Basic formula</th>
<th>meq/liter</th>
<th>Additions</th>
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<tbody>
<tr>
<td>Na⁺</td>
<td>140</td>
<td>Glucose—usually 15%</td>
</tr>
<tr>
<td>K⁺</td>
<td>40</td>
<td>FreAmine—usually 40% †</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>1.66</td>
<td>Insulin—as specified</td>
</tr>
<tr>
<td>Cl⁻</td>
<td>90</td>
<td>Glucagon—as specified</td>
</tr>
<tr>
<td>SO₄⁻</td>
<td>1.66</td>
<td></td>
</tr>
<tr>
<td>Acetate*</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>HCO₃⁻</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>Chloramphenicol†</td>
<td></td>
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</tr>
</tbody>
</table>

* In later experiments (most of Fig. 3) HCO₃⁻ was omitted and acetate was increased to 90 meq/liter, with no detectable effect upon the experimental outcome.
† Chloramphenicol was added to provide 20 mg/rat per 24 hr. Amoxicillin was included in the infusion mixture in early experiments, but subsequently was given instead as a single intramuscular injection (15 mg) at the end of the operation.

TABLE 2. Hepatic DNA synthesis at intervals after partial hepatectomy in eviscerated rats with and without insulin treatment

<table>
<thead>
<tr>
<th>Rats</th>
<th>DNA dpm/10 µg*</th>
<th>Proportion of rats with dpm &gt;400†</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>A. Normal</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Nonhepatectomized 161 ± 12</td>
</tr>
<tr>
<td></td>
<td></td>
<td>24-hr hepatectomized 5537 ± 520</td>
</tr>
<tr>
<td></td>
<td></td>
<td>48-hr hepatectomized 1966 ± 71</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B. Eviscerated, insulin-treated</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Nonhepatectomized 181 ± 24</td>
</tr>
<tr>
<td></td>
<td></td>
<td>24-hr hepatectomized 782 ± 275</td>
</tr>
<tr>
<td></td>
<td></td>
<td>48-hr hepatectomized 1633 ± 231</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C. Eviscerated, insulin-deprived</td>
</tr>
<tr>
<td></td>
<td></td>
<td>48-hr sham 100 ± 43</td>
</tr>
<tr>
<td></td>
<td></td>
<td>48-hr sham 1088 ± 304</td>
</tr>
</tbody>
</table>

Group A—Normal control rats.
Group B—Evisceration at the time of partial hepatectomy. Infusion mixture contained insulin (0.5–1.0 U/kg per 24 hr) and glucose (5–10%).
Group C—Evisceration at time zero, sham operation or partial hepatectomy at 18–22 hr, termination at 66–70 hr (i.e., 48 hr after the second operation). Infusion mixture contained 5% glucose, no insulin. The hepatectomized rats in Group C significantly exceeded the sham and nonhepatectomized controls of Groups C and B in rate of DNA synthesis (P < 0.05 and < 0.01, respectively, by t test), but do not differ significantly from the 48-hr hepatectomized rats that received insulin in Group B (P < 0.2).

Mean value ± SEM.
† Ratio of number of rats having greater than 400 dpm/10 µg of DNA to total number of rats in group. No nonhepatectomized controls in these experiments have ever exceeded 400 dpm/10 µg.

To determine the rate of DNA synthesis, 50 µCi of [methyl-³H]thymidine, specific activity 6 Ci/mmol (Schwarz/Mann, Orangeburg, N.Y.), were injected intravenously. One hour later animals were disconnected from the infusion assembly and anesthetized with ether, blood samples were withdrawn for glucose (Dextrostix) (12) and insulin (15) determinations, and livers were excised; slices were fixed for histological study, and the remainder was frozen for subsequent assay of DNA specific activity (12).

RESULTS AND DISCUSSION

In normal rats, hepatic regeneration induced by partial hepatectomy follows a well-known course; the rate of DNA synthesis, which serves as a convenient and dependable index of regenerative activity, starts to increase at around 14 hr after the hepatectomy, reaching an initial peak at about 24 hr (1). Data for normal control rats for the present experiments are shown in Table 2, Group A, for comparison with Group B, which is composed of eviscerated rats given insulin and glucagon (but no glucose). Although an appreciable elevation in DNA synthesis occurred in Group B, it was considerably delayed, and rose to only a third of the normal 24-hr maximum by 48 hr.

In Group C (Table 2) insulin was omitted altogether. So that endogenous pancreatic hormone levels would be minimal, the rats in this group were eviscerated on the day before the hepatectomy; they received only the basic salt mixture plus 5% glucose throughout the 3-day experimental period. At 48 hr after the hepatectomy (and 68 hr after evisceration), the rate of DNA synthesis had attained a level only a little below the value obtained in the insulin-treated animals in Group B; according to statistical analysis this difference is not significant. Serum insulin levels determined at the termination of the experiment averaged 1.3 ± 0.4 U/ml, which is not significantly different from 0 by the technique employed; in comparison the mean level for normal portal vein serum insulin was 62 ± 6 U/ml. Glucagon is reported to disappear from the blood even more rapidly than insulin (16). Hence, although trace amounts of endogenous insulin (and glucagon) could still have been present in these animals (Group C), it seems unlikely that these hormones could serve as sole determinants of hepatic regeneration. Nevertheless, the sluggishness of regeneration in their absence signaled their importance, even if only in an ancillary role.

We accordingly infused insulin and glucagon, either singly or combined, in increasing amounts into partially hepatectomized eviscerated rats. The results are shown in Fig. 1, in which insulin dosages are displayed along the abscissa, and glucagon along the ordinate. All rats were killed 24 hr after evisceration and hepatectomy, at which time animals given only the basic salt formula with or without added glucose and amino acids showed no rise in hepatic DNA synthesis (Fig. 1, bottom left). As shown in Table 2 (Group C), moderate regenerative activity does occur in eviscerated rats by 48 hr after partial hepatectomy in the absence of hormone treatment. Hence a large increase in DNA labeling rate at 24 hr would indicate both an acceleration and an enhancement of regeneration—i.e., a more rapid initiation of DNA replication involving a larger population of cells.

As seen along the abscissa in Fig. 1, addition of insulin to the infusion mixture had only a modest effect, as did glucagon by itself, as seen along the ordinate. When the two hormones were
Fig. 1. Effect of continuous infusion of insulin and glucagon on hepatic DNA synthesis determined 24 hr after partial hepatectomy in eviscerated rats. Rats were eviscerated, partially hepatectomized, and infused for 24 hr with the dosages of insulin and glucagon indicated on the graph. Vertical bars represent DNA labeling in rats receiving either commercial grade (solid bars) or crystalline (striped bars) glucagon and/or insulin. Numbers above each experimental group indicate number of rats; each bar represents a single animal. FreAmine (usually 40%) was added when glucagon was present, and glucose (15%) when insulin was present, except that glucose was omitted when low doses of insulin were combined with doses of glucagon sufficient to maintain blood sugar levels.

Groups E, E', and G (Fig. 2) responded impressively to the combined hormone treatment, even though it was not begun until 6-7 hr after the partial hepatectomy, when early events of regeneration are known to be already underway. The same treatment was without effect in eviscerated rats whose livers remained intact (F and H), implying that insulin and glucagon are not by themselves prime initiators of hepatic cell proliferation.

Consistent with the finding that delaying the hormone treatment in eviscerated rats did not impair its effectiveness, was the observation that in normal rats after partial hepatectomy the portal vein serum insulin level fell abruptly from $62 \pm 6 \mu U/ml$ to near zero within 2 hr, with a variable, slow return towards normal (17). An increased supply of insulin appears not to be required during early regeneration.

Either continuous (Fig. 1) or delayed (Fig. 2) infusion of insulin and glucagon, in combination, was capable of restoring DNA biosynthetic activity in eviscerated rats to levels found in normal animals at the same time interval (24 hr) after partial hepatectomy (see Table 2), although the results were variable and not all rats responded fully. The considerable variability among individual rats, evident in Figs. 1 and 2, is not surprising in view of the drastic nature of the experimental procedure; perhaps in some animals the flow of hepatic arterial blood may be insufficient to sustain active liver growth. At least partly because of the variability, optimal dosages can only be defined within broad limits, and no maximally effective ratio of insulin to glucagon appears obvious; the ratio may not be critical.

As noted under Fig. 1, administration of insulin was generally accompanied by glucose, and glucagon by FreAmine. We have not fully evaluated the effects of FreAmine in these...
experiments, but have observed that considerable enhancement of DNA synthesis can occur even in its absence, when insulin, glucose, and glucagon are present.

Evidence from surgical laboratories, mentioned earlier, implicates insulin, or insulin plus glucagon, as regulatory influences in hepatic growth (2, 3, 5, 10). In further support of the participation of glucagon is a report by Short et al. (6) of its function in a mixture containing also triiodothyronine, amino acids, and heparin, that was found capable of initiating DNA synthesis in intact rats. On the other hand, glucagon had a suppressive effect upon DNA synthesis in primary cultures of fetal rat hepatocytes stimulated by insulin (18), but in vitro conditions probably do not accurately reproduce those existing in vivo. Recently, Price and his coworkers have reversed their original view of glucagon as an inhibitory influence (5). They have now found that simultaneous administration of insulin and glucagon to eviscerated, partially hepatectomized rats shortens the pre-replicative period (11), as confirmed by our present data; under the conditions they employed, the rise in DNA synthesis was advanced to 48–72 hr from beyond 96 hr after partial hepatectomy.

We have evaluated the effects of insulin and glucagon separately in eviscerated animals, and demonstrated that both hormones are indeed required for effective enhancement of the regenerative response to partial hepatectomy. In appropriate doses the effects of insulin and glucagon combined are capable of restoring DNA synthesis to normal, even though the hepatic blood supply is reduced to the fractional volume provided only by the hepatic artery. The relative importance of volume as opposed to composition of portal blood in augmenting liver growth has long been controversial. Our observations underscore the significance of the quality of blood supplied.

Insulin is widely regarded as an anabolic, growth-promoting hormone, in contrast to glucagon, which, at least in the liver, has opposing actions, particularly upon carbohydrate and lipid metabolism, where it exerts a catabolic influence (19, 20). Our findings imply, however, that in promoting hepatic regeneration the two hormones act, not in opposing ways, but rather in synergy. The main function of the glucagon in this instance could be to maintain glucose supplies in the presence of high insulin levels (21); however, other known effects such as stimulation of amino-acid transport (22), activation or induction of specific enzymes or proteins (23–25), or phosphorylation of specific proteins (26, 27), could be critical for hepatocyte proliferation. Somewhat in favor of the latter possibilities is our finding that insulin, when administered with large amounts of glucose, and in the demonstrated absence of hypoglycemia, is by itself ineffective in accelerating regeneration in eviscerated rats.

The observations that significant regenerative activity is inducible in the near total absence of pancreatic hormones, and that delayed administration of insulin and glucagon is equally effective as continuous treatment in restoring regeneration to normal in eviscerated rats, both suggest that, in spite of the pronounced influence of these two hormones, one or more additional factors are required to initiate cell proliferation. Further findings consistent with this view are the abrupt fall in portal vein insulin levels in normal rats shortly after partial hepatectomy, and the failure, so far, of insulin and glucagon to appreciably activate DNA synthesis in eviscerated, non-hepatectomized rats. Although agents other than insulin and glucagon may function in initially activating regeneration, the synergistic action of these two hormones appears to be a major influence in regulating the rate and perhaps also the extent of the growth process.

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