

421

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GROWTH-STIMULATING FACTOR IN REGENERATING CANINE LIVER

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Summary Extracts from dog livers which had been regenerating for 24, 48, and 72 h after hepatectomy were infused for 6 h into the left portal vein of animals which had fresh portacaval shunts (Eck fistula) and which were killed 2 and 3 days later. The brief exposure to the 48-h and especially the 72-h regenerating liver extracts induced a delayed proliferative response predominantly in the left liver lobes, with a slight spillover effect to the right liver lobes but none to the kidney. The response reached its peak 3 days later. In the left but not the right liver lobes, both the 48-h and the 72-h regenerating liver extract reversed the atrophy ordinarily caused by Eck fistula in 3 days and partly prevented the ultrastructural hepatocyte deterioration characteristic of Eck fistula. The active liver extracts apparently contained a growth-control factor or factors which is (are) not insulin or glucagon.

Introduction

THERE has been much recent interest in the role of so-called portal hepatotrophic substances in regulating or permitting hepatic regeneration.¹ In this communication, the additional, and by no means contradictory, possibility is considered that something in the liver frag-

ment after hepatectomy can contribute to, or even initiate, its own regrowth. The question was examined in a previously described Eck fistula model² which permitted extracts of normal and regenerating liver to be introduced into one of the tied-off portal branches and tested for their regional as well as general hepatic effects.

Methods

Healthy adult mongrel dogs were used. Anaesthesia for all procedures and for killing was sodium pentobarbitone supplemented with phencyclidine hydrochloride ('Sernylan') and succinylcholine chloride ('Anectine').

Complete portacaval shunt (Eck fistula) in dogs causes a trebling of hepatocyte proliferation and a halving of hepatocyte size within 4 days.² Because information about the evolution of these changes within the first 4 days was required to interpret the results of our experiments, control animals had a large side-to-side portacaval shunt with individual ligation of the right and left portal branches, and were killed from $\frac{1}{2}$ to 4 days later (table I). In most of the experiments (tables II and III), a catheter was placed in the ligated left portal branch and led through a subcutaneous tunnel to an infusion pump.² Variations from this technique and differences in the substance infused are summarised in tables II and III.

Killing was timed exactly from completion of the shunt. The liver extract or control infusion was started 4 to 6 h after the portacaval shunt. All infusions were 6 h in duration with two exceptions: in groups 7-9 intraperitoneal infusions were given over 5 min; and in group 17, a left portal branch infusion was given for 3 days.

The method of LaBrecque and Pesch³ was used to prepare a cytosol extract from blood-free normal adult livers and from liver fragments remaining after 72% hepatectomy⁴ done 1, 2, and 3 days previously. In essence, extraction in the cold from 100 g of the asanguineous liver specimen was by preliminary homogenisation, plus saline dilution, secondary centrifugation for 15 min at 10 000 g, and final ultracentrifugation for 2 h at 140 000 g.

For infusion, about 75 ml of a reddish-yellow, clear supernatant was obtained; its activity is heat-stable⁵ but destroyed by perchloric acid. The extract contained soluble proteins and other cytoplasmic constituents and was free of organelles, cell membranes, microsomes, and virus by electron microscopy. Insulin,⁶ and glucagon⁶ in the supernatant from normal dog livers and 72-h regenerating livers were measured in the laboratory of Dr R. H. Unger (Dallas, Texas). The extract was given on the day of preparation.

All experimental end points were histopathological, as previously described,^{8,9} the studies being done without knowledge of the experimental group from which the specimens came. 2 h before killing, 0.2 mCi/kg of body-weight ($\text{CH}_3\text{-}^3\text{H}$) thymidine (47 Ci/mmol) was given intravenously. Liver tissues from the right and left lobes and from a kidney were fixed in 10% buffered formaldehyde. Other liver samples were fixed in glutaraldehyde solution, postfixed in osmic acid, and embedded in epoxy resin.

Frozen and paraffin sections were prepared from the formalin-fixed material. The frozen sections were stained for fat and paraffin sections were stained with haematoxylin and eosin and other special stains. The size of hepatocytes in the mid-lobular zones was measured⁸ and the results expressed in arbitrary size units. The hepatocytes in the middle zone of the liver lobules were also used for measuring in electronmicrographs the area of rough endoplasmic reticulum per volume of cytoplasm.¹⁰ Hepatocytes and glomerular cells in mitosis were counted.

Other paraffin sections of liver and kidney were dewaxed, dipped in Ilford K2 nuclear emulsion, and used for autoradiography (exposure for 4-8 weeks until counts of the labelled nuclei stopped increasing). With liver sections, only hepatocytes were counted, excluding stromal and other cells. In the

TABLE I—ACUTE CHANGES IN LEFT LIVER LOBES CAUSED BY PORTACAVAL SHUNT IN DOG LIVERS

Group	Time (h) after shunt	No. labelled hepatocytes per 1000 hepatocytes (mean \pm s.d.)	Mitoses per 1000 hepatocytes (mean \pm s.d.)	Cell size units (mean \pm s.d.)
Normal (10)	0	1.61 \pm 0.39	0.07 \pm 0.03	0.173 \pm 0.013
A (2)	12	1.60 \pm 0.14	0.08 \pm 0.01	0.170 \pm 0.002
B (2)	24	1.55 \pm 0.35	0.06 \pm 0.01	0.177 \pm 0.003
C (2)	36	1.55 \pm 0.07	0.08 \pm 0.01	0.176 \pm 0.006
D (6)	48	1.92 \pm 0.78	0.07 \pm 0.02	0.151 \pm 0.009*
E (6)	72	3.20 \pm 0.48†	0.14 \pm 0.04*	0.123 \pm 0.017†
F (2)	96	4.15 \pm 0.07†	0.18 \pm 0.03†	0.086 \pm 0.000†

There were no significant differences between right and left lobes in any of the groups.

* $P < 0.01$; † $P < 0.001$ when compared with left liver lobes in normal dogs.

kidney samples, only labelled glomerular endothelial, mesangial, and epithelial cells were recorded.

Results

Acute Effects of Eck Fistula

Hepatocyte thymidine incorporation had not significantly increased by 2 days after Eck fistula nor had the number of mitoses, but by 72 h they had doubled and by 96 h they had almost trebled (table 1).

Within 12 h after the Eck fistula, the amount of rough endoplasmic reticulum in the hepatocytes was already decreased and there was disruption and dilation of the cisternæ. The amount of glycogen and the number of membrane-bound ribosomes in the cells were also reduced. Some of the mitochondria were enlarged and the cristæ distorted. The Kupffer cells were enlarged and contained ceroid, hæmofuscin, and some hæmosiderin. All these changes were progressive and by 48 h there was statistically significant hepatocyte atrophy (table 1). At 72 h these findings were more advanced and by 96 h the liver cells had shrunk to half their original size (table 1) and they were irregular in shape, depleted of glycogen, and contained a little fat. The lobules were reduced in size and there was condensation of the reticulin frame-

work around the central veins. These structural alterations were similar to those previously reported.²

All the changes following Eck fistula were equal in the right and left lobes. For experiments with liver-extract infusion, the dogs with Eck fistula of 48-h (group D) and 72-h duration (group E) served as controls against which additional effects could be judged over the same periods (tables II and III).

Infusions and 48-h Eck Fistula Liver

The abnormalities characteristic of a 48-h Eck fistula liver were not altered by a 6-h infusion into the left portal branch of 75 ml saline (group 2), extract from normal livers (group 3), or extract from 24-h regenerating livers (group 4) (table II). However, a deviation from the expected pattern was identifiable when extract from 48-h (group 5) and 72-h regenerating livers (group 6) was infused. 72-h extract was most potent, nearly quadrupling the rate of thymidine incorporation and mitosis in the directly infused left lobes (table II). There was no spillover effect in the right lobes. Furthermore, the extracts from 24, 48, and 72-h regenerating livers had no effect upon either liver side when given intraperitoneally over 5 min or by a systemic vein over 6 h.

None of the infusions had any significant effect upon the beginning atrophy of the 48-h Eck fistula liver (table II). By light and electron microscopy the right and left lobes appeared the same.

Infusions and 72-h Eck Fistula Liver

The cell renewal in 72-h Eck fistula livers was unchanged by infusions into the left portal vein of 75 ml saline (group 12) or extracts from normal (group 13) and 24-h regenerating livers (group 14) (table III). In contrast, there was a statistically significant proliferative response after similar 6-h infusions of extracts from 48-h regenerating livers (group 15), and an even greater response to extracts of 72-h regenerating livers (group 16) (table III). The resultant doubling of thymidine incorporation and more than trebling of mitoses was largely confined to the directly infused left liver lobes, but a statistically significant small increase was also seen on the right

TABLE II—EFFECT OF REGENERATING-LIVER EXTRACTS ON LIVER ATROPHY AFTER 2 DAYS OF ECK FISTULA

Group no.	Treatment	No. labelled hepatocytes/1000 hepatocytes		Mitoses/1000 hepatocytes		Cell size units	
		Left	Right	Left	Right	Left	Right
1	No infusion (6)	1.92 \pm 0.78	1.78 \pm 0.61	0.07 \pm 0.02	0.08 \pm 0.03	0.151 \pm 0.009	0.150 \pm 0.009
2	Saline (2)	1.85 \pm 0.35	1.90 \pm 0.57	0.08 \pm 0.01	0.08 \pm 0.02	0.152 \pm 0.014	0.153 \pm 0.015
	L.P.V. liver extract						
3	Normal (6)	1.85 \pm 0.39	1.88 \pm 0.23	0.09 \pm 0.03	0.09 \pm 0.02	0.149 \pm 0.012	0.150 \pm 0.019
4	24-h regeneration (6)	1.90 \pm 0.55	1.92 \pm 0.59	0.09 \pm 0.02	0.08 \pm 0.02	0.148 \pm 0.009	0.149 \pm 0.014
5	48-h regeneration (6)	3.85 \pm 2.71*	1.80 \pm 0.40	0.19 \pm 0.13†	0.08 \pm 0.01	0.158 \pm 0.014	0.152 \pm 0.010
6	72-h regeneration (6)	6.97 \pm 0.79†	1.82 \pm 0.53	0.35 \pm 0.04†	0.09 \pm 0.02	0.149 \pm 0.008	0.149 \pm 0.011
	I.P. liver extract						
7	24-h regeneration (2)	1.90 \pm 0.42	1.85 \pm 0.21	0.10 \pm 0.04	0.10 \pm 0.01	0.151 \pm 0.005	0.150 \pm 0.001
8	48-h regeneration (2)	1.85 \pm 0.07	2.00 \pm 0.14	0.08 \pm 0.00	0.08 \pm 0.01	0.152 \pm 0.014	0.153 \pm 0.009
9	72-h regeneration (2)	1.95 \pm 0.21	2.05 \pm 0.21	0.09 \pm 0.04	0.10 \pm 0.04	0.154 \pm 0.008	0.155 \pm 0.008
	I.V. liver extract						
10	48-h regeneration (2)	1.92 \pm 0.16	1.89 \pm 0.31	0.09 \pm 0.01	0.08 \pm 0.03	0.149 \pm 0.004	0.150 \pm 0.001

L.P.V.: left portal vein infusion lasting 6 h; I.P.: intraperitoneal infusion lasting 5 min; I.V.: intravenous (systemic vein) infusion lasting 6 h. All infusions given 4–6 h after shunt.

Group 1 was control group D in table I.

* $0.05 < P < 0.1$ left vs right in each group;

† $0.05 < P < 0.1$ left vs right in each group and left vs left of group 1; ‡ $P < 0.001$ left vs right in each group and left vs left of group 1.

TABLE III—EFFECT OF REGENERATING-LIVER EXTRACTS ON LIVER ATROPHY AFTER 3 DAYS OF ECK FISTULA

Group no.	Treatment	No. labelled hepatocytes/1000 hepatocytes		Mitoses/1000 hepatocytes		Cell size units	
		Left	Right	Left	Right	Left	Right
11	No infusion (6)	3.20±0.48	3.12±0.73	0.14±0.04	0.15±0.04	0.123±0.017	0.121±0.017
12	Saline (2)	3.50±0.14	3.40±0.42	0.14±0.06	0.13±0.05	0.128±0.002	0.128±0.002
	<i>L.P.V. liver extract</i>						
13	Normal (8)	3.18±0.61	3.20±0.58	0.14±0.03	0.15±0.03	0.121±0.016	0.129±0.012
14	24-h regeneration (6)	3.02±0.74	2.93±0.64	0.14±0.04	0.15±0.05	0.125±0.016	0.127±0.019
15	48-h regeneration (6)	5.72±2.92*	3.00±0.75	0.27±0.14*	0.15±0.05	0.169±0.011†	0.122±0.013
16	72-h regeneration (8)	11.73±3.38†§	3.86±0.67	0.62±0.19†‡	0.20±0.04	0.182±0.027†	0.121±0.019
17	72-h regeneration (6)	10.77±3.00†	3.37±0.62	0.59±0.15†	0.17±0.04	0.171±0.024‡	0.123±0.014

L.P.V.: left portal vein infusion lasting 6 h except in group 17 (3 days). All infusions given 4–6 h after shunt.

Group 11 was group E in table I.

*0.05 < P < 0.1 left vs right in each group;

†P < 0.001 left vs right in each group and left vs left of group 11;

‡P < 0.01 left vs right in each group and left vs left of group 11;

§0.05 < P < 0.1 right vs right of group 11;

¶P < 0.05 right vs right of group 11.

side when the 72-h regenerating liver extract was infused (group 16). When the 72-h regenerating liver extract was given over the full 3 days of observation instead of the early 6 h (group 17), the effect on the left side was almost as great (table III). With both the 48-h and 72-h regeneration extract (groups 15–17), the differences between the two liver sides were significant.

Both the 48-h and 72-h regenerating liver extracts (groups 15–17) significantly arrested the atrophy of the 72-h Eck fistula liver in the left lobes but not on the right side (table III) and, in fact, these left lobar hepatocytes were almost as large as or even larger than those in the 10 normal dogs summarised in table I. By light microscopy, the left lobar hepatocytes contained less glycogen than normal, but more than in the atrophic right lobar hepatocytes. The Kupffer cells in both lobes were enlarged and contained ceroid and lipofuscin. Ultrastructurally, the left lobar hepatocytes were almost normal. Glycogen granules were frequent and the cisternæ were normal but the area of rough endoplasmic reticulum per volume of cytoplasm and the number of membrane-bound ribosomes were slightly reduced and the number of small fat vacuoles was increased. There were no obvious differences between the right lobar hepatocytes and those in the livers of the dogs in group E controls.

All statistical analyses for tables I–III were based on Student's *t* test. By the Wilcoxon and Wilcoxon non-parametric analysis of variance of ranks, the results for labelled hepatocytes in the important group 16 were significant ($P < 0.01$).

Renal Changes

The frequency of mitoses in the glomerular cells and the number of these cells incorporating thymidine neither varied significantly between the various groups of experimental animals nor differed from the findings in the kidneys of the normal dogs.

Hormones in Liver Extract

Non-specific binding of 18–20% in the glucagon assay was the same in cytosol from the 8 normal and the 7 regenerating livers. The corrected glucagon concentrations were 70.5 ± 53.24 (s.d.) and 108.43 ± 68.48 (s.d.) pg/ml, respectively.

Insulin was not detectable in any of the specimens. Non-specific binding averaged 36.5% in the cytosol from regenerating livers compared with 4–5% in cytosol from normal livers. This reflected a greater destruction of the insulin isotope tracer by the regenerating cytosol.

Discussion

The idea of intrinsic hepatic growth control factors was stated in reports, which we have recently reviewed,¹⁰ dating back almost 50 years. The earliest convincing evidence was provided by Teir and Ravanti¹¹ and Blomqvist¹² who found stimulatory activity in a crude mash of weanling and regenerating rat livers but not in mash from intact adult livers. Levi and Zeppa¹³ supported the concept of a liver factor but their work was not reproducible.^{14 15} However, LaBrecque and Pesch³ strengthened Blomqvist's contentions in a different kind of rat experiment in which they used the same liver extract as in our experiments rather than a mash. In the meanwhile, the reviewed evidence^{10 16} from many laboratories suggested that a stimulatory factor appeared in the serum after partial hepatectomy. In rats, Morley and Kingdon¹⁷ characterised this serum substance as a heat-stable protein with a molecular weight of about 26 000. LaBrecque and Pesch³ speculated that liver and serum factors might be the same, a hypothesis advanced 17 years earlier by Blomqvist¹² but never firmly established because of many contradictory reports.

In our experiments, the Eck fistula and unilateral portal branch infusion model to test liver extract activity was chosen for two reasons. First, the exceptional reproducibility of this system had previously been established,² making it easy to identify experimentally induced mitotic increases as compared with control results. Second, the Eck fistula liver has a higher than normal rate of resting hepatocyte mitoses.^{2 18} Thus, if mitosis-inhibiting substances had been present in any of the extracts, an effect might have been unmasked. Inhibitory serum or liver factors have been claimed or speculated about,^{3 16 19–22} but none were found in this study, possibly because the background mitotic activity was not great enough.

In contrast, stimulatory activity which was not present in extracts from normal livers or 24-h regenerating hepatic remnants, became demonstrable in the 48-h

remnants and even more in extracts from tissues that had been regenerating for 72 h. The mitotic response in the left lobes of Eck fistula livers after a 6-h exposure to active extracts did not become evident until almost 2 days later and after this it increased for at least another day. By this time, there was a very minor mitotic increase in the non-infused contralateral liver lobes. No doubt, it is more than coincidence that both full development of extract potency from regenerating liver as well as the full response to it in a second animal required about 3 days. This is the time when hepatic regeneration in dogs reaches a peak.⁴ The lack of changes in the kidneys suggested liver specificity, but to rule out extra-hepatic effects, other experiments will be necessary in which active liver extracts are infused directly into suspect target organs.

Proliferation was not the only effect. The hepatocyte atrophy and ultrastructural deterioration of Eck fistula, which was not altered by the active extracts for the first 2 days after their infusion, reversed between the second and third days in the directly infused left lobes. The result by 3 days was the appearance of healthy cells of normal or even supernormal size which were proliferating at an increased rate.

The identity of the growth factor(s) in regenerating liver is speculative. The fact that it cannot be found in liver remnants until regeneration is far advanced reduces the possibility that it is an uncomplicated initiator. Yet, its probable importance as a biological determinant is underscored by the fact that its effects far outlast a shortlived administration.

Continuous intraportal infusions of insulin have been shown to influence the Eck fistula liver in much the same way as the liver extracts of the present report.² However, there was no evidence that unaltered insulin, or for that matter glucagon, were the active ingredients in the cytosol. Whether the hepatic factor is non-hormonal or some known or unidentified hormone, its role in regeneration eventually will have to be reconciled with the demonstration that canine liver regeneration is virtually eliminated by extirpation of all the non-hepatic splanchnic organs including the pancreas.^{8, 23} Is the genesis of the factor we have described an independent quality of the partially resected liver which creates a new environment in which the collective splanchnic hepatotropic substances can be effective, mainly by complex hormone interactions?^{7, 8, 16, 23, 24} Or is development of the liver factor dependent from the outset on the splanchnic hepatotropic substances including insulin? Preparation and testing of extracts from non-regenerating post-resection liver remnants in animals subjected simultaneously to evisceration should clarify this issue.

Other important steps in this inquiry will be to isolate and identify the active substance(s) in the liver extracts, using standard biochemical techniques and to determine if the activity is organ and species specific. The accurate testing of purified moieties for their hepatic effect also should be possible with the model used in this study. The clinical application of such research could be promotion of repair of the acutely damaged liver which has failed to respond with appropriate regeneration.

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SURVIVAL OF HEART ALLOGRAFTS IN RATS TREATED WITH AZATHIOPRINE AND SODIUM SALICYLATE

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Summary In a rat heart transplant model representing a severe mismatch, median survival time (M.S.T.) with no treatment was 6 days. M.S.T. with azathioprine was 6 days, and with azathioprine and methylprednisolone 7 days. Azathioprine with promethazine hydrochloride gave an M.S.T. of 15 days. In rats treated with sodium salicylate alone M.S.T. was 16 days, and when azathioprine was administered for 12 days in a group continuously treated with sodium salicylate, all hearts were beating normally at 50 days.

Introduction

PROMETHAZINE hydrochloride has an immunosuppres-