The Role of Hepatotrophic Factors in Liver Regeneration—
A Brief Review Including a Preliminary Report
of the In Vitro Effects of Hepatic
Regenerative Stimulator Substance (SS)

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INTRODUCTION

The mammalian liver possesses a remarkable capacity to regenerate in response to
a variety of stimuli. Following removal of the median and left lateral lobes in the rat
(68% hepatectomy), DNA synthesis rises sharply at 16–18 hours with mitosis
reaching a maximum at 28 hours. The remaining lobes nearly double in size by 48
hours and within seven days approach the weight of the normal liver, at which time
growth ceases as abruptly as it began [1]. This regenerative activity involves both
hypertrophy and hyperplasia of cells and, while many studies have characterized
the time sequence as well as the morphological and biochemical changes occurring in this
process [1,2,3], the mechanism(s) which so precisely regulate this remarkable growth
spurt have remained elusive. Understanding the control of growth at the cellular level
has obvious implications for the elucidation of both normal and malignant growth,
and liver regeneration provides an excellent model with which to study this problem.

In earlier studies I have demonstrated that an extract of weanling rat liver (SS) will
produce a 21/2-fold increase in incorporation of tritiated thymidine (3HTdr) into
hepatic DNA [4,5,6] when injected intraperitoneally into a one-third hepatectomized

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adult rat. This represents a true increase in DNA synthesis and not merely an effect on thymidine pool size because a similar increase in mitotic index is also observed. There is a lag period of 12–15 hours between the injection of SS and its effect on DNA synthesis. Extracts from adult rats produce no such stimulation. However, the same extract prepared from a 68% hepatectomized rat produces a fourfold stimulation of DNA synthesis. In the hepatectomized adult rat, SS appears prior to the first wave of DNA synthesis, remains present while DNA synthesis is increased, and is no longer detectable when DNA synthesis returns to normal.

Preliminary characterization of the factor has shown it to be present in the soluble cell fraction only (145,000 g × 2 hour supernatant); it is resistant to heating at 100°C for 15 minutes, is inactivated by perchloric acid treatment, and is precipitated, but not inactivated, by ethyl alcohol. Dialysis increases its activity and its molecular weight is approximately 10,000, as determined by ultrafiltration through Amicon membranes.

Under a controlled lighting schedule, both DNA synthesis and SS activity in the weanling donor rats follow the same distinct diurnal rhythm [7]. Reversal of the lighting schedule reverses the rhythm of DNA synthesis but SS activity no longer correlates with the peak of DNA synthesis. Thus it seems unlikely that SS is the major control factor in normal DNA synthesis although it clearly stimulates regenerative growth. Barbiroli and Potter [8], by controlling periods of light and food availability, demonstrated the existence of at least two different regulatory systems for DNA synthesis in the regenerating rat liver. DNA synthesis was maximal 23 hours after hepatectomy, regardless of the time of day hepatectomy was performed. However, this was superimposed upon a background rhythm of endogenous DNA synthesis which was controlled by the light-dark and feeding schedule. SS appears to be primarily involved with the former and not the latter mode of DNA synthesis.

To the extent that it has been tested, SS appears to be organ specific, affecting liver but not bone marrow, kidney or spleen. It is not species specific, however, since SS prepared from weanling rat stimulates DNA synthesis in both regenerating rat liver and normal non-regenerating mouse liver [6].

All of my previous experiments were in vivo studies and required large amounts of SS [4,5,6,7]. Thus in 1971 work was begun on the development of a culture system to maintain isolated adult hepatocytes in the non-proliferating state as an in vitro system to study SS. The present paper reports preliminary results of these experiments. In addition, a brief review of hepatotrophic factors is presented in order to evaluate the possible significance of SS in relation to other known factors.

METHODS

Preparation of SS

Weanling male Sprague Dawley rats, 60–90 gm in weight, were used as the source of SS. Animals were housed in a temperature controlled room, acclimatized to a fixed lighting schedule (light 6 AM–6 PM) for one week, and fed ad libitum.

SS was prepared by removing weanling rat livers under light ether anesthesia between 7 AM and 8 AM and homogenizing them in cold 0.9% sodium chloride (35% weight to volume) in a Sorval omni-mixer. The homogenate was heated at 65°C for 15 minutes followed by centrifugation at 27,000 × g and 4°C for 20 minutes. The supernatant was added to 6 volumes of ice cold ethanol, stirred for 2 hours in the cold and centrifuged at 27,000 × g and 4°C for 20 minutes. The precipitate was redissolved in water, centrifuged and the supernatant saved. This procedure yields about an 80-
fold purification of the extract with respect to protein content and SS is stable for at least 6 months when lyophilized and kept at \(-20^\circ C\). All preparations of SS were tested in the standard \textit{in vivo} assay [4] to confirm activity before use \textit{in vitro}.

\textit{In Vitro Studies}

Tissue slices were prepared by hand with a Stadie Riggs tissue slicer (Arthur Thomas and Co., Philadelphia, PA) and incubated in Krebs Ringer Bicarbonate medium under an atmosphere of 95\% \(O_2\)–5\% \(CO_2\) as previously described [4]. Isolated liver parenchymal cells were prepared by an enzymatic method based on that of Howard and Pesch which utilizes collagenase and hyaluronidase to separate the cells. Details of the method along with some more recent improvements are described elsewhere [9].

\textit{Culture}

Isolated liver parenchymal cells were prepared as described [9] except that all utensils and media were sterilized and careful sterile technique was utilized throughout. Cells were added to 60 mm Falcon plastic petri dishes at an initial concentration of 0.5–1.0 \(\times 10^6\) cell/ml, 3 ml per plate. Media were changed at 4 hours, 24 hours, and 72 hours and plates were kept in a humidified, \(CO_2\) incubator with 5\% \(CO_2\).

\textit{DNA Synthesis}

Tritiated thymidine (\(^3\)HTdr, 20–60 Ci/mmol) was incubated with slices, cell suspensions, or cultures for one–two hours in different experiments. DNA was isolated by the method of Morley and Kingdon [10] and an aliquot added to Aquasol (N.E. Nuclear Corp., Boston, MA) for measurement of isotope incorporation into DNA. Additional aliquots were utilized to quantitate total DNA by the diphenylamine method of Burton [11]. Results are expressed dpm/mcg DNA or total acid precipitable counts/plate. The latter were obtained by washing cells two times with isotope free medium and two times with ice cold 0.6 N perchloric acid. The precipitate was heated with 0.6 N perchloric acid at 80\(^\circ\)C for 15 minutes to solubilize the DNA for ease in counting.

\textit{Materials}

Culture media were obtained from Flow Laboratories, Inc. (Rockville, MD), or Microbiological Associates (Walkersville, MD). Tritiated thymidine (\(^3\)HTdr) was from New England Nuclear Corp. (Boston, MA). HI-WO\(_3\)-BA\(_{2000}\) was obtained from International Scientific Industries, Inc. (Cary, IL).

\textbf{RESULTS}

\textit{Culture of Liver Parenchymal Cells}

The major factor responsible for the recent success in culturing liver parenchymal cells, both in this lab and others [9,12,13], has been the improvement in the technique of preparing isolated cells for culture. No cells, other than parenchymal cells, have been demonstrated in our preparation by light or electronmicroscopy, thus allowing one to attempt culture with a pure parenchymal cell suspension. This avoids the overgrowth of contaminating fibroblasts which has been such a problem in the past [14] by eliminating them prior to culture. In addition, this method routinely produces cells with 95–98\% viability, as determined by exclusion of trypan blue, with intact ultrastructure, retained intracellular glycogen and potassium, and high rates of
endogenous respiration [9]. Although it has been possible to prepare successful cultures with cell preparations having lower viabilities and less retention of normal ultrastructure, the ability to repair these functions in vitro is limited and overall plating efficiency and long term viability were depressed. In addition, they carried out normal hepatocyte functions less well [9,15].

Method

All attempts at spinner culture or slant tube culture failed as nearly 100% of the cells were dead within 24–48 hours. Monolayer culture was successful in Leighton tubes with cover slips, plastic and glass petri dishes, and T-flasks. Plating efficiency was better in plastic and currently we routinely use 15 × 60 mm plastic petri dishes (Falcon or Corning). Optimal plating concentration appears to be 0.6–1.0 × 10^6 cells/ml with 3 ml/plate. With our best culture conditions (see below), a plating efficiency of 50–75% is obtained. Cells remain viable for 5–7 days with stable morphology, cell numbers and DNA concentration/plate.

Characteristics

Within 30–60 minutes of plating, most viable cells are firmly attached and by 5 hours have begun to flatten and collect in groups of 3–5 cells. By 24 hours cells are flat and polygonal in shape and are arranged in larger groups of 10–30 cells. At 48 hours large collections of cells are apparent and form an almost confluent sheet on the plate (Fig. 1). Ultrastructure is normal and tight junctions and bile canaliculi reform (Fig. 2). ³H-uridine and ¹⁴C-leu are readily taken up into RNA and protein, respectively, and the cells release albumin into the culture medium as demonstrated by specific immune precipitation in octerlony plates. Control medium incubated with fetal calf serum but no hepatocytes showed no precipitin lines. (Specific rabbit anti-rat albumin was obtained from Cappel Laboratories Inc., Downington, PA.) In contrast, very little ³H-thymidine is incorporated into DNA and cells have not been observed in mitosis.

![Liver parenchymal cells after 72 hours in culture. An almost uniform monolayer may be seen in this phase contrast photomicrograph. Several crenated, dead cells (C) may be seen overlying the surface of the monolayer. The picture represents a magnification of 100 times.](image-url)
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FIG. 2. Higher power phase contrast view of liver parenchymal cells after 72 hours in culture. Bile canaliculi (B) may be clearly seen between adjacent parenchymal cells. The picture represents a magnification of 430 times.

Media

Hepatocytes did not appear to be very discriminating with respect to medium requirements and Hams F12, Hams F10, Dulbecco-Vogt's modification of the minimal essential medium of Eagle (DV-MEM), HI-WO₂-BA₂₀₀₀ of Jenkins, Medium 199, and Rosewell Park Institute Medium 1630 all supported hepatocytes in monolayer culture. However Hams F12, DV-MEM, and Medium 199 produced the best viability and plating efficiency and Hams F12 is used routinely at present.

Serum

Fetal calf serum was necessary for cellular attachment to plastic or glass culture plates. Concentrations as low as 2-5% would suffice, but 10% appeared to be optimal. Concentrations greater than 10% did not improve plating efficiency. Cells did not attach in the absence of serum and viability dropped rapidly after 24 hours. In HI-WO₂-BA₂₀₀₀ cells would survive without serum, but at 24 hours were still rounded and only lightly attached and could be easily released by agitation of the plate. Within 48 hours, cells were firmly attached and flattened although overall plating efficiency and size of groups was smaller.

Supplements

Dexamethasone has been reported to be necessary for survival of fetal liver cells in culture and to suppress fibroblast growth [16]. In our system dexamethasone appeared to decrease the cellular aggregation into large sheets and also led to multiple vacuoles within the hepatocytes. No improvement in plating efficiency or survival was noted and fibroblasts were never observed in culture with or without dexamethasone so it is no longer used.

Pyruvate improves the retention of glycogen and potassium by liver cells during their enzymatic preparation [17]. Addition of 0.1 mM pyr to our standard media produced only minor increments in plating efficiency and viability and is no longer performed routinely.

Insulin produced a dramatic improvement in plating efficiency and viability.
Increasing concentrations from 5 mU/ml to 100 mU/ml produced progressive improvement but higher concentrations were of no further benefit.

Arginine is synthesized by the liver and arginine-free medium has been used to suppress fibroblast contamination since fibroblasts cannot synthesize arginine [18]. DV-MEM without arginine supported hepatocytes in culture fairly well although not as well as the complete medium. Again, no fibroblasts were observed with either medium and there appears to be no benefit in deleting arginine from the medium with the present technique of cell preparation.

Current Method

Based on the above observations, routine culture at present involves preparation of isolated liver parenchymal cells with 0.05% collagenase and 0.10% hyaluronidase in Ca++ and Mg++free MEM of Eagle. 0.5–1.0 × 10⁶ cell/ml are plated in 15 × 60 mm plastic petri dishes, 3.0 ml/plate. Cells are cultured in Ham's F12 with 10% fetal calf serum, 50 units each of penicillin and streptomycin per ml, and 80 mU insulin/ml. Medium is changed at 4 hours to remove unattached, dead cells and again at 24 and 72 hours. Studies are usually performed between 24 and 72 hours.

In Vitro Effects of SS

Addition of SS in concentrations of 0.5–10% to the incubation medium of liver slices or isolated liver cell suspensions has no effect on incorporation of ³HTdri into DNA. This is not surprising since these are, by the limitations of the techniques, short-term experiments of 2–4 hours and I have previously shown that there is a 12–15 hour lag between the injection of SS and its effect in vivo [4].

In preliminary experiments adding SS to the culture medium at the time of plating, a dose-dependent increase in uptake of ³HTdri into DNA is seen at 21 hours. At 46 hours, even the lowest concentration of 0.2% (0.2% by volume represents 8 μg extract protein per ml medium) produces nearly a fourfold increase (Table 1).

In studying the time course of this stimulation, SS was added in 1.0% concentration when the medium was changed at 48 hours. No increase was seen at 6 hours but at 12 hours incorporation was almost doubled in the treated cultures (Fig. 3). Thus, as in the in vivo experiments, there is a distinct lag between the time of exposure to SS and appearance of its effect.

Preliminary screening of the effects of SS on other cells in culture (Table 2) reveals no significant increased incorporation of ³HTdri into acid precipitable counts when SS is added to normal human or mouse lymphocytes in the presence of phytohemag-

| %SS  | 21h     | 46h     |
|------|---------|---------|
| 0.0  | 13.8 ± 2.9 | 67.8 ± 21 |
| 0.2  | 18.4 ± 1.8 | 229 ± 55 |
| 20.0 | 53.3 ± 14  | 362 ± 89 |

SS was added to cultures at the time of plating (0 hr). 5 ×CiPi HTdri was added at the times noted and cells harvested 1 hour later and incorporation of ³HTdri into DNA determined. Each result represents the mean ± SEM of 3 culture plates. At 21 hours only the mean counts in the 20% SS plates are significantly different from 0% controls (p <0.05). At 48 hours both the 0.2% (p <0.05) and 20% (p <0.025) are significantly different from controls as determined by student's t test [59].
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TIME COURSE OF SS STIMULATION OF DNA SYNTHESIS IN LIVER PARENCHYMAL CELLS IN CULTURE

FIG. 3. Isolated adult rat parenchymal cells in primary culture. Medium was changed after 48 hours of culture and SS added to some of the cultures as denoted by the broken line and open circles. Each point is the mean of 3 culture plates. The values at 12 hours ($p < 0.0025$) and 18 hours ($p < 0.05$) are significantly different from each other as determined by student's $t$ test [39]. Ordinate refers to total time in culture.

glutinin, L 1210 murine leukemia cells or Y1 mouse adrenal cells. However, MH1C1 rat hepatoma cells showed an almost twofold increase in acid precipitable counts 24 hours after exposure to 0.2% SS in culture (11,137 cpm vs. 6930 cpm). Increasing the concentration of SS to 2.0% produced 40,891 acid precipitable counts per minute at 24 hours, an almost sixfold increase.

The HTC cell line, another rat hepatoma, also responded to low concentrations of SS in a screening experiment. Thus the apparent specificity of SS in vivo appears to be retained in vitro as well.

DISCUSSION

Various factors including species, age, magnitude of resection, environment, and diet modify the regenerative response; however, none of these appears to exercise

| Stimulates                  | No Effect                               |
|-----------------------------|-----------------------------------------|
| Primary rat liver culture   | Lymphocytes (human, mouse)              |
| Hepatoma                    | Adrenal (Y1—mouse)                      |
| MH1C1 (rat)                 | Leukemic (L1210—mouse)                  |
| HTC (rat)                   | Glial tumor (C6, rat)                    |
|                             | Ovary (CHO, hamster)                     |
primary control [1,3]. Interest in humoral control of liver regeneration has waxed and waned for several decades and many poorly controlled experiments have yielded contradictory results. Over the past 15 years, however, careful experiments have clearly implicated humoral factors in the control of liver regeneration: (1) Leong et al. [19], Sigel, Acevedo, and Dunn [20] and others [3] showed that partial hepatectomy stimulated DNA synthesis in liver autografts remote from the site of hepatectomy. In addition Sigel et al. [21,22] showed that the hepatocytes stimulated to synthesize DNA were clustered around the portal vein if the autograft was connected to systemic blood via the portal vein, and around the central vein if the direction of flow was reversed. This dependency on the direction of blood flow within the autograft is consistent with the results of earlier studies showing that liver regeneration begins periportally in the normal regenerating liver [23,24,25]; (2) Moolten and Bucher [26] and Sakai [27] performed meticulous cross-circulation experiments with parabiotic rats and observed an increase in DNA synthesis in the unresected partner when the other rat was partially hepatectomized.

While these experiments appear to confirm the involvement of a humoral factor or factors in the control of liver regeneration, no such substance has been isolated; furthermore, these experimental results do not differentiate between the release of a stimulator or the decrease of a circulating inhibitor. Nor has it been ascertained what organ or tissue might be the source of such factors.

Various fractions of liver have been employed experimentally in attempts to stimulate or inhibit liver regeneration but results have not been consistent [28,29,30,31,32,33]. Similar problems have arisen in attempts to stimulate regeneration with serum from regenerating animals (see [1] for review). However, Morley and Kingdon [34] have recently demonstrated the appearance of a protein in rat serum 12 hours following partial hepatectomy which will stimulate DNA synthesis in a recipient animal. Interestingly their serum extract has a molecular weight similar to that of SS, is also stable to heat at 100° C, and appears in the serum at a time following partial hepatectomy similar to that at which SS becomes demonstrable in the liver extract of regenerating, adult rats [4]. Whether the substances are identical will require further investigation.

The above studies do not establish the source of the stimulator(s) and do not exclude the possibility of coexisting inhibitors. Kuo and Yoo [35], Verly [36], Miyamato [37], Otsuha [38] and Lavigne et al. [39] have presented evidence for hepatic chalones (inhibitors) and my early experiments demonstrated inhibition of DNA synthesis by extracts from older rats in contrast to the stimulation produced by the same extract from younger or regenerating rats [4,32]. Thus, there may be an interplay of stimulator(s) and inhibitor(s) with the final results determined by this interaction.

The exact nature of these factors remains a mystery. Mann [40] first demonstrated the importance of portal blood in liver regeneration in 1931. Starzl et al. confirmed these results by showing that an auxiliary liver graft, if deprived of splanchic blood, underwent severe atrophy [41] even though it had an equal volume of flow from the systemic circulation. If the graft was perfused with splanchic blood it thrived, but the recipient's own liver atrophied [42]. Marchioro et al. [43,44] showed the same effects on the normal liver by use of a partial (split) porta caval transposition in which one portion of the liver received systemic venous blood and the other portal blood. The side perfused by vena caval blood atrophied while the other side hypertrophied.

The existence of portal factors was further confirmed in elegant experiments
carried out by Fisher et al. [45], with parabiotic rats. Partial hepatectomy in one animal increased DNA synthesis in the liver of the normal partner. Partial hepatectomy followed by portacaval shunt prevented the increase in DNA synthesis in the operated liver but further augmented DNA synthesis in the non-operated partner, suggesting the presence of a portal factor which reached the normal partner's liver in increased concentration by bypassing the operated liver.

A series of experiments by Starzl et al. strongly implicated insulin as the portal factor. In split portal perfusion experiments, part of the liver received the effluent from pancreas, duodenum, stomach, and spleen; the other lobes received their blood from the intestine. Those receiving intestinal blood atrophied and those with pancreaticoduodenal blood hypertrophied [46]. When alloxan diabetes or pancreatic atrophy was superimposed on the above experiment, with insulin supplied subcutaneously and thus equally to both sides, most of the differences were abolished. In more direct experiments, insulin, glucagon, or combinations of the two were directly infused into the portally deprived lobe. Only insulin prevented the atrophy [47,48]. Orloff and co-workers have also confirmed the presence of a portal factor [49] and its probable origin in the pancreas [50]. However, whereas crude pancreatic extract induced "spectacular" regeneration, insulin alone did not [50].

Price et al. [51,52,53] have shown that the dog liver will regenerate in the total absence of abdominal organs, in fact an absolute increase in mitotic rate occurred compared to hepatectomized animals whose abdominal organs were intact. IV glucagon returned this response to normal. However, all animals were also receiving endogenous or peripheral insulin.

In further studies, Price and co-workers [54] and Bucher and Swaffield [55,56] demonstrated that rat liver would also regenerate in the absence of splanchnic organs, although at a much slower rate than in the intact rat. Price found that insulin decreased DNA synthesis in a dose dependent fashion with large doses greatly delaying DNA synthesis. Glucagon increased the magnitude of DNA synthesis and produced an earlier peak in a dose dependent manner. However, the best response was found with an appropriate insulin/glucagon ratio. Bucher and Swaffield found that neither insulin nor glucagon was very effective alone but that in combination they completely restored regenerative activity. Similarly, insulin and glucagon together, but neither by itself, protected mice from fulminant murine hepatitis. However, Bucher and Weir [57] have consistently failed to induce hepatic proliferation by administering insulin and glucagon to normal or eviscerated animals. Thus, while pancreatic factors appear important as facilitators of liver regeneration, they are probably not the primary activator of liver growth, and the search for such factors continues.

The development in several labs [58], particularly those of Bissel [12] and Bonney [13], of reliable methods for maintaining normal adult liver parenchymal cells in non-replicating culture provides a powerful new tool for the investigation of putative regenerative factors. Preliminary results suggest that these cells will provide a useful assay system for SS. In vitro SS appears to retain not only its specificity for liver cells but also the lag period observed before its effect in vivo. The small amounts of SS required for an effect will allow the use of standard biochemical techniques in the further purification of SS and characterization of its mode of action and interaction with other hepatotrophic factors.

Work on SS to date indicates that it not only augments DNA synthesis in the already regenerating liver, but also induces it in the non-regenerating liver. Based on
studies of its diurnal rhythm, SS does not appear to control normal endogenous DNA synthesis. Thus it may be specific for regenerative or rapid growth, as is further suggested by its presence in weanling rats and in adult rats following hepatectomy but its absence from normal adult liver. It is organ specific but species non-specific. Its specificity in increasing only DNA synthesis and the consistent lag period before its effect is seen both in vivo and in vitro suggests that it acts early in the sequence of events leading to DNA synthesis. The same lag period suggests that it is not simply increasing the rate of DNA synthesis through a mass action effect. Its apparent molecular weight of about 10,000, failure to cross a dialysis membrane, and precipitation by ETOH also argue against its being a simple, low molecular weight, building block or cofactor. The development of a reliable in vitro assay for SS should allow the purification and identification of SS and elucidation of its mode of action.

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