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Altered Acinar Distribution of Glutamine Synthetase and Different Growth Response of Cultured Enzyme-positive and -negative Hepatocytes after Partial Hepatectomy[1]

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ABSTRACT

Partial hepatectomy (PH) results in the persistent drop of the specific activity of glutamine synthetase (GS) (EC 6.3.1.2). This drop correlates with the reduced proportion of GS$^+$ hepatocytes and with the reduced GS$^+$ area surrounding the central veins such that GS$^+$ hepatocytes are arranged in a single cell layer only.

Cultivation of hepatocytes isolated at various times after PH revealed considerable differences in the growth characteristics of GS$^+$ and GS$^-$ hepatocytes discriminated by immunocytochemistry. In the absence or presence of epidermal growth factor and insulin, the labeling index of GS$^-$ hepatocytes peaked in cultures established 48 h after PH at 10% and 50%, respectively, while that of GS$^+$ cells was much lower (2% and 6%). In cultures established at later times after PH the labeling index of GS$^-$ cells decreased gradually, while that of GS$^+$ hepatocytes increased continuously, reaching about 20% and more than 50% for controls and epidermal growth factor/insulin-treated cultures, respectively, in cultures established 72 after PH. Norepinephrine stimulated the labeling index of both cell populations during the first 24 h only, but again GS$^-$ hepatocytes responded somewhat earlier than did GS$^+$ hepatocytes. These results demonstrate that the differences in the growth characteristics of GS$^+$ and GS$^-$ hepatocytes are due to different priming of these cells *in vivo* and may result in the different expansion of the respective cell populations during regeneration after PH.

INTRODUCTION

After PH,[3] the liver quickly regains its lost mass. This regenerative growth proceeds in a characteristic temporal and spatial pattern, *i.e.*, in several sequential waves of DNA synthesis and mitosis starting in the periportal and ending in the pericentral zone (1–5; for further references, see Ref. 6). There is considerable evidence that this compensatory growth involves almost all hepatocytes which replicate at least once, rather than just a specialized population of "stem cells" (7–9). Nevertheless, it appears that periportal hepatocytes may be more active than the pericentral ones. However, the reasons for this different response of hepatocytes from different locations within the acinus and for the coordinate temporal pattern of their entry into S phase are largely unknown.

There is now accumulating evidence that GS is a marker enzyme for a small perivenously localized subpopulation of hepatocytes (GS$^+$ cells) in the rat (10–13) as well as in other species (13, 14). The heterogeneous distribution of the GS$^+$ cells is strikingly stable throughout life (15) and does not change in response to various physiological conditions, such as starvation or changing diets (11, 16). Changes in the localization of GS are noted only in response to some special cases of experimental liver injury affecting pericentral hepatocytes and are all characterized by a reduction of the enzyme-positive area, *i.e.*, the number of GS$^+$ hepatocytes (11, 16, 17). Reduction was also noted for streptozotocin-induced diabetes (12). Recently, the GS$^+$ hepatocytes have been shown *in vitro* to possess growth characteristics markedly different from those of GS$^-$ cells (6, 18). These differences may have substantial impact on the expansion of the respective cell populations during regenerative growth.

In the present study we have determined the localization of GS in liver parenchyma during regeneration after partial hepatectomy by using immunohistochemistry. In particular, we have addressed the questions of whether changes in the distribution of GS are reflected in changes of the pool size of the GS$^+$ hepatocyte subpopulation and of GS activity. Furthermore, we have characterized the effect of partial hepatectomy and subsequent regeneration on the differential growth response of GS$^+$ and GS$^-$ hepatocytes in primary culture under various growth conditions.

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats, weighing 210 to 320 g, were kept for at least a week in a controlled dark/light cycle on a standardized diet of Alma and water *ad libitum*.

Animal Treatment and Preparation of Liver Sections. Partial hepatectomy was performed by removing two-thirds of the rat liver (19) modified as described previously (20). At various times thereafter the animals were anesthetized with sodium pentobarbital (60 mg/kg, i.p.), and the residual livers were flushed with saline via a cannula in the portal vein. Parts of the livers were then removed for preparation of homogenates, and the remainder was fixed by perfusion with 3.5% paraformaldehyde in buffered saline for 10 min, then excised, cut into small cubes, and immersed in the same fixative for 1 h (in some experiments for 2 h). For immunohistochemistry, the pieces of tissue were then embedded in paraffin and sectioned. Alternatively, cryotome sections were prepared as described (10).

Isolation and Cultivation of Hepatocytes. Hepatocytes from whole livers were isolated as described previously (18). The isolated hepatocytes were suspended in Williams Medium E containing 2 mM glutamine (6) before being added at a final density of 25,000 cells/cm^2 to collagen-coated (21) six-well plates (Greiner, Nürtingen, Federal Republic of Germany). All cultures were maintained for 48 h in the presence of 10^{-7} M dexamethasone. Optionally, EGF (20 ng) and insulin (10^{-7} mol) were added to the culture medium as indicated in the figure and table legends.

Autoradiography and Scintillation Counting. In experiments assessed by autoradiography or scintillation counting, [^{3}H]thymidine (specific activity, 40 to 60 Ci/mmol) was added to the culture medium during the period from 24 to 48 h at a concentration of 10 or 5 mCi/ml, respectively. Scintillation counting was performed as described (18). For autoradiography, cells were fixed with 10% buffered formalin, followed by 98% ethanol and mounting with glue on microscopic slides. Further processing including prior immunocytochemical staining for GS was performed as described recently (18).

Immunohistochemical Techniques. Immunocytochemical localization of glutamine synthetase by a modification of the unlabeled antibody peroxidase-antiperoxidase method of Sternberger *et al.* (22) was performed as described in detail by Gebhardt *et al.* (16, 17). The primary

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[3] The abbreviations used are: PH, partial hepatectomy; GS, glutamine synthetase (EC 6.3.1.2); EGF, epidermal growth factor.

antiserum used was a rabbit anti-rat liver glutamine synthetase antiserum diluted 1:500 with phosphate-buffered saline.

Image Analysis of Immunohistochemically Stained Sections. Liver sections stained by the peroxidase-antiperoxidase method for glutamine synthetase were subjected to image analysis using a μBAC 8810 image analyzer (Grude Elektronik, München, Federal Republic of Germany) connected with a Sony CCD videocamera. In order to determine the size of the area staining for glutamine synthetase, whole liver sections were scanned with a microscopic window of approximately 0.75 mm^2. The microscopic pictures were converted to binary images representing the GS-positive area in black and the remainder in white. The threshold for conversion to black was 5 gray values above background level on a scale of 200 gray values. This procedure led to the determination of the size of the stained area with an error of less than 10% when all black pixels were counted and expressed as the percentage of the total pixels covering the microscopic window. For statistical analysis, between 45 and 212 independent measurements were performed.

Enzymatic Assays and Analytical Procedures. For determination of enzyme activities in the liver, tissue homogenates (1:10 in the respective assay buffers) were prepared with the aid of a Dounce glass homogenizer. These initial homogenates were further diluted with the same buffers to concentrations suited for the different enzyme assays and were additionally homogenized with a Branson sonifier (15 s) (16).

Glutamine synthetase (50 mM Tris buffer, pH 7.4) was measured either by the γ-glutamyltransferase assay (23) or with the radiochemical assay described (10). Protein was determined by the method of Lowry *et al.* (24) using bovine serum albumin as a standard.

Statistics. Differences between means were calculated by Student's *t* test. Only values of *P* lower than 0.01 were considered to be significant.

RESULTS AND DISCUSSION

As known for many years (25), two-thirds partial hepatectomy was found to result in a rapid decline of GS specific activity, reaching a new level at about 56% of the initial value after 3 days (Table 1). As revealed by immunohistochemistry on paraffin sections prepared at various times after PH, this drop in enzyme activity was associated with the reduction of the GS^+ cell layer surrounding the terminal hepatic venules from the normal size of one to 3 rows of cells (10) down to one row of cells only which, in some cases, was not even continuous (Fig. 1). This slightly changed localization, which could be quantified by use of image analysis (Table 1) as a reduction of the GS^+ area by about 40% within the first 48 h, persisted for at least 3 wk (Fig. 1). If hepatocytes were isolated at various stages of the regenerative process and stained for GS, a similar decrease in the percentage of GS^+ hepatocytes was found (Table 1). Thus, there was a good correlation between the reduction of GS activity, the size of the parenchymal area staining for GS, and the number of GS^+ hepatocytes at all times after PH.

Table 1 *Alterations of GS activity and of the population size of the GS^+ hepatocytes during regeneration following PH*

Time after PH[a]	Specific activity of GS (milliunits/mg of protein)[b]	Relative no. of GS^+ hepatocytes (%)[c]	Size of GS^+ area (% of total area of sections)[d]
0 h	325 ± 57[e] (5)[f]	7.8 ± 0.7	3.74 ± 1.41 [184][g]
4 h	337 ± 66 (3)	7.3 ± 0.6	3.25 ± 1.29 [50]
24 h	198 ± 43 (3)	6.4 ± 0.7	1.63 ± 0.82[h] [52]
48 h	162 ± 48 (4)[h]	2.8 ± 0.3[h]	1.37 ± 1.08[h] [45]
72 h	127 ± 46 (3)[h]	2.1 ± 0.2[h]	0.91 ± 0.77[h] [62]
120 h	151 ± 63 (3)[h]	ND[i]	1.18 ± 0.91[h] [54]
3 wk	139 ± 52 (3)[h]	ND	1.09 ± 0.68[h] [50]

[a] Zero time values refer to controls.
[b] Determined with the γ-glutamyltransferase assay (23).
[c] Calculated on the basis of 1000 to 2000 hepatocytes cultured for 4 h.
[d] Determined with the μBAC 8810 image analyzer as described in "Materials and Methods."
[e] Mean ± SD.
[f] Numbers in parentheses, number of independent measurements.
[g] Numbers in brackets, number of total measurements.
[h] Statistical evaluation (different from controls): $P < 0.001$.
[i] ND, not determined.

This slight but persistent change in the localization of GS is remarkable for several reasons: (a) it contrasts with the pronounced alterations found for other hepatic enzymes, *e.g.*, pyruvate kinases (26); (*b*) it is characterized by the principal restriction of the GS^+ hepatocellular phenotype to the perivenous endplate which is also maintained in many other types of liver injury (12, 16, 17, 27); and (*c*) it contrasts with the regenerating process after intoxication with CCl_4 where the original number of GS^+ hepatocytes is regained (27, 28).

In order to learn more about possible alterations in the (relative) size of the hepatocyte population carrying the GS^+ phenotype we studied the growth potential of GS^+ and GS^- hepatocytes at various times after PH. This growth potential has recently been characterized *in vitro* to be strikingly different for the two phenotypes (6, 18). The experimental approach used herein closely followed the design of Francavilla *et al.* (29), but included the immunocytochemical distinction between GS^+ and GS^- hepatocytes in conjunction with autoradiographic detection of [^{3}H]thymidine-labeled nuclei recently introduced for characterizing the differential growth characteristics of GS^+ and GS^- hepatocytes (6, 18).

Hepatocytes isolated from rat livers at different times after PH and cultured in the presence of dexamethasone only (*i.e.*, the absence of growth factors) for 48 h showed relatively low labeling indices when labeled with [^{3}H]thymidine during the second 24-h period (Fig. 2). However, whereas the labeling index of GS^- hepatocytes showed a transient maximum at about 10% after 24 h, that of GS^+ was still below 2% at this time but steadily increased thereafter, resulting in an inverse proportion after 72 h. Thus, at that time, the labeling index of GS^+ cells was almost 20% compared with about 4% for GS^- cells.

In the presence of insulin and EGF at optimal concentrations (Fig. 2), cultured hepatocytes from control rats showed a marked difference in the growth response reported previously (18). Again, the labeling index of GS^- hepatocytes increased in cultures established within the first 24 h after PH, reaching nearly 50%, and declined in those established later (Fig. 2). In contrast, the labeling index of GS^+ hepatocytes remained well under 10% during the first 24-h period but then increased dramatically during the following 48 h. In cultures established 72 h after PH, the labeling index of GS^+ hepatocytes was well comparable to that of GS^- cells at their peak response (Fig. 2). In cultures exposed to EGF only, a qualitatively similar response could be observed compared with matched cultures in the presence of insulin and EGF (compare Fig. 2 and Table 2).

With respect to the GS^- cell population which comprises more than 90% of the hepatocytes, these data confirmed the results of Francavilla *et al.* (29) who found a maximal growth response in cultures established 24 h after PH. The GS^+ cell population, on the other hand, responded much later, in both the absence or presence of insulin and EGF. However, the fact that the GS^+ hepatocytes may reach (although at later times) an at least comparable extent of DNA synthesis as GS^- hepatocytes indicates that the growth potential of these cells is not generally lower as predicted by the concept of a "streaming liver" (30), which is based on a gradual increase of senescence of the hepatocytes toward the central veins and does not encompass (two) distinct hepatocellular phenotypes.

In contrast, the delayed entry into S phase of the GS^+ hepatocytes found *in vivo* and *in vitro* (18) seems to be a characteristic feature of this hepatocellular phenotype and, thus, also a consequence of the position of these cells at the

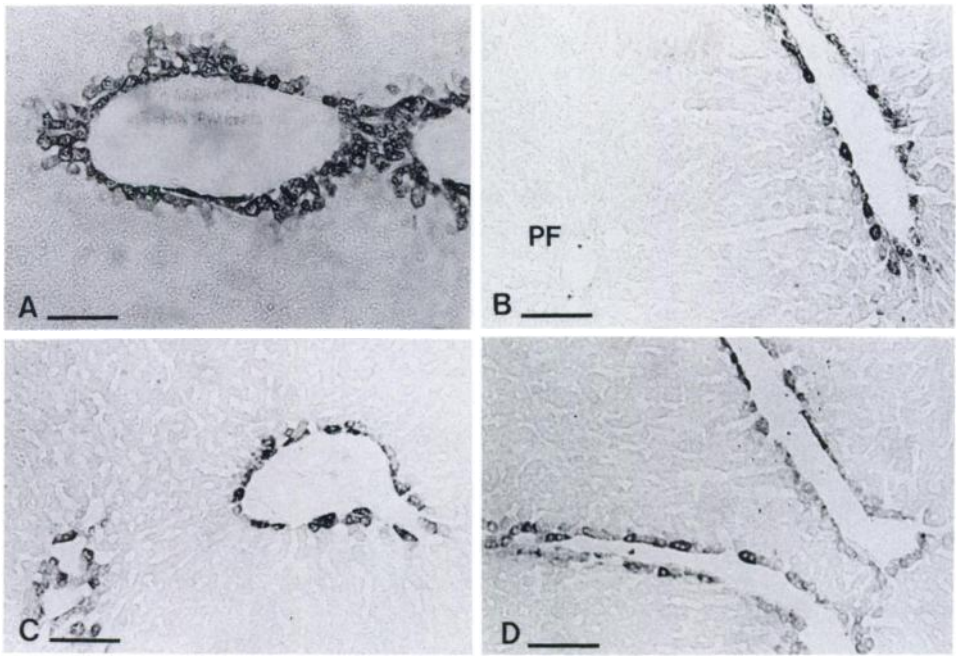

Fig. 1. Immunohistochemical localization of glutamine synthetase in liver sections at various times after partial hepatectomy. *A*, paraffin section of control liver with GS⁺ hepatocytes surrounding a central vein. *B*, section obtained 18 h after PH. Note the reduced size of the GS⁺ area (*PF*, portal field). *C*, section obtained 36 h after PH, two central veins. *D*, liver section 3 wk after PH, two branching central veins. *Bars*, 100 μm. × 125.

Table 2 *Effect of norepinephrine on the labeling index of GS⁺ and GS⁻ hepatocytes at different times after PH*

Hepatocytes isolated at various times after PH were cultured for 48 h, exposed to [³H]thymidine from 24 to 48 h, and processed for immunocytochemistry for GS and for autoradiography.

Time after PH (h)	Labeling index (%)			
	EGF (50 mg/ml)		10^{-4} M norepinephrine/EGF	
	GS⁻[a]	GS⁺	GS⁻	GS⁺
0	23.5 ± 2.1[b]	2.2 ± 0.3	48.6 ± 5.4[c]	7.7 ± 1.0[c]
4	25.7 ± 2.7	2.0 ± 0.4	53.5 ± 6.1[c]	13.8 ± 1.9[c]
12	29.0 ± 2.8	3.4 ± 0.3	41.4 ± 4.5[c]	19.3 ± 2.4[c,d]
24	37.1 ± 4.7[d]	4.9 ± 0.8	39.6 ± 3.2	10.2 ± 1.3[c]
48	25.1 ± 3.0	12.6 ± 1.1[d]	26.5 ± 2.8	13.8 ± 1.7[d]
72	20.4 ± 2.9	38.2 ± 4.4[d]	22.7 ± 2.5	36.9 ± 3.9[d]

[a] Discriminated by immunocytochemical staining for GS.
[b] Mean ± SD, determined on the basis of 300 to 500 cells for each cell population.
[c] Statistical evaluation (different from the matching culture with EGF only): $P < 0.01$.
[d] Statistical evaluation (different from zero time, controls): $P < 0.01$.

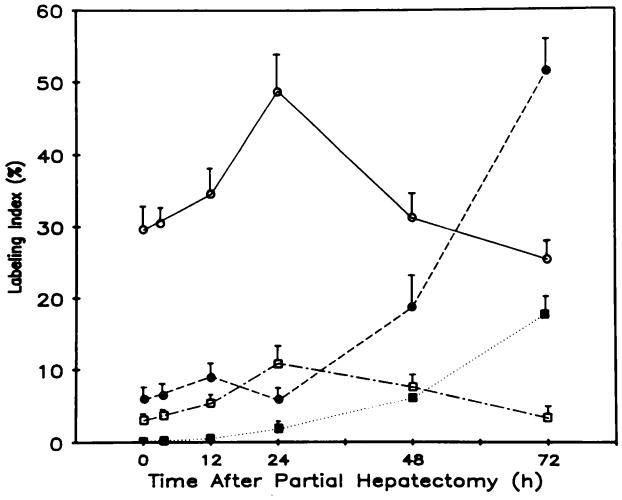

Fig. 2. Labeling index of GS⁺ (*closed symbols*) and GS⁻ hepatocytes (*open symbols*) determined in primary cultures established at various times after PH. All cultures were maintained for 48 h in the presence (○, ●) or absence (□, ■) of EGF and insulin and were exposed to [³H]thymidine during the period from 24 to 48 h. Thereafter cultures were processed for immunocytochemistry and autoradiography as described in "Materials and Methods." *Points*, mean; *bars*, SD.

very end of the acinus in Zone 3 (6). Furthermore, it fits well with the general observation *in vivo* that hepatocytes located around the central veins enter S phase much later than do periportal cells (2, 5) and suggests that these GS⁺ hepatocytes are particularly primed by an as yet unknown stimulus as DNA synthesis progresses in a wave-like fashion toward the central vein. It is possible that EGF itself or, as recently proposed (31), transforming growth factor α may be involved in this priming process.

Since norepinephrine, *i.e.*, stimuli that act via α_1-receptors, may play an important role during the early phase of regeneration (32) we were particularly interested in its influence on the GS⁻ and GS⁺ hepatocyte populations. If norepinephrine was added together with EGF [an essential condition (32)], an immediate response of GS⁻ hepatocytes was observed in controls or in cultures established 4 h after PH, while the response of the GS⁺ hepatocytes was most prominent in cultures established 12 h after PH (Table 2). For both cell populations, however, a significant influence of norepinephrine was restricted to the early period after PH, and no effect could be noted after 48 h or later. These results demonstrate that both the strength and also the time point of the entry of GS⁺ hepatocytes in the S phase are dependent on the types of growth stimuli.

Taken together, the results on the growth stimulation may lead to an attractive hypothesis for the diminution of the number of GS⁺ hepatocytes during regeneration from partial hepatectomy. Because of their rapid growth response a much larger proportion of the GS⁻ cells than of the GS⁺ cells may be involved in the regenerating growth during the first 48 h. Since inhibiting factors which may aid in terminating regeneration (33, 34) most probably increase in parallel to the increasing liver mass, the GS⁺ hepatocytes are exposed to a much less favorable environment for growth when they reach their most sensitive period. As a consequence this cell population will not enlarge to the same extent as will GS⁻ hepatocytes within the period necessary for the completion of the original liver mass, leading to a smaller fraction of these cells after regeneration.

A necessary precondition for this explanation is that there is no change in the phenotype with respect to GS expression. This condition seems to hold as long as the hepatocytes reside in their proper environment, *i.e.*, unchanged liver architecture, since the GS^+ phenotype, once established, seems to be quite stable under many physiological and experimental conditions including hepatocarcinogenesis (35, 36). Only if the environment of the hepatocytes is completely changed, *e.g.*, after transplantation into fat pads (19), during growth stimulation *in vitro* (18), or by cocultivation (37), may a reprogramming of gene expression (optionally aided by a mitotic cycle) occur, resulting in the shift of the GS^- to the GS^+ phenotype or vice versa. These phenomena point to the existence of an as yet unidentified positional signal in the liver parenchyma responsible for the phenotypic expression and/or exact localization of the GS^+ hepatocytes. Although unlikely, an alternative explanation for the persistent reduction of the GS^+ area would thus be that PH somehow causes a persistent reduction of that signal in spite of the almost completely maintained acinar architecture.

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