Control of Growth and Expression of Differentiated Functions of Mature Hepatocytes in Primary Culture

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ABSTRACT. Since methods to disperse and culture hepatocytes were developed 15 years ago, numerous investigations have shown that primary cultures of mature hepatocytes retain most liver functions and respond as well to various hormones as those *in vivo*. Thus they are the most suitable system *in vitro* for studies on the liver. Moreover, recently it was found that differentiated hepatocytes in culture can grow under certain conditions and that this growth is regulated not only by several hormones, such as insulin, epidermal growth factor and serum growth factor, but also by a cell membrane factor and proteins in the environmental matrix through cell contact.

This article describes the biochemical characterization of regulatory factors for hepatocyte growth and functions and their reciprocal expression. The mechanisms of liver regeneration, differentiation and carcinogenesis and the importance of the tissue architecture for these events are discussed mainly on the basis of our findings.

I. Introduction

Great efforts have been made over many years to establish conditions for culture of hepatocytes without loss of their diverse liver functions, since so called "established lines of liver cells" do not show appreciable liver functions or respond to hormones (8).

Of the six types of cells found in the liver (parenchymal hepatocytes, bile canalicular cells, Kupffer cells, Ito cells, endothelial cells and fibroblasts), parenchymal cells are responsible for most liver functions. Parenchymal hepatocytes also constitute about 70% of the total cell population in liver. Therefore, the first step in culture of functional hepatocytes was to isolate these parenchymal cells free from other cell types. This was achieved by Berry and Friend in 1969 by perfusion of the liver with collagenase (1) and their method was later improved by Seglen (34). Thus hepatocytes with various functions became available in high yield as an almost pure population.

For use of these cells in studies on liver functions, the next step was to find culture conditions in which they maintain their hormonal responses for a relatively long

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Abbreviations used: WE, Williams medium E; EGF, epidermal growth factor; *cis*-OH-Pro, *cis*-hydroxyproline; PDGF, platelet derived growth factor; HTP, hepatotropin; KD, kilodalton; TAT, tyrosine amino transferase; SDH, serine dehydratase; TO, tryptophan 2,3-dioxygenase; G6PD, glucose-6-phosphate-dehydrogenase; ME, malic enzyme; MEM, minimal essential medium; CSM, cell surface modulator.

Fig. 1. Phase-contrast microscopic appearance of hepatocytes of adult rats in primary culture. Hepatocytes at confluency were cultured in Williams medium E supplemented with 5% calf serum and 10^{-9} M dexamethasone for 1 day (A, $\times 140$) and 14 days (B, $\times 210$).

time. Suitable culture conditions were first reported by Bissell *et al.* in 1973 (2). Since then there have been many reports showing that this primary culture system is the most suitable for studies on liver functions and hormonal responses *in vitro* (2, 3, 6, 9, 25, 28, 30, 37).

A drawback of these culture conditions has been that mature hepatocytes in primary culture do not proliferate at all, even in the presence of fetal bovine serum, which usually stimulates growth of established cell lines. Therefore, in these culture conditions hepatocytes could survive for only about a week and then became detached from the culture dishes, being replaced by non-parenchymal epithelial cells, which contaminated a few percentage in the original parenchymal cell population and could grow under these conditions (Fig. 1). These epithelial cells do not show any liver functions and so called "established liver cell lines" probably originate from them.

Therefore, not only for culture *per se*, but also for studies on the mechanisms of liver regeneration and hepatocarcinogenesis, it was important to establish good conditions for growth of primary cultures of hepatocytes that allow the cells to maintain liver functions and hormonal responses. Here we describe recent findings on the growth and functions in primary culture of hepatocytes and discuss their regulatory mechanisms.

II. Growth regulation of mature hepatocytes

1. *Cell density-dependent regulation of growth.* Mature hepatocytes usually re-

main in the resting phase (G_0) *in vivo*. However, unlike nerve or muscle cells, hepatocytes can proliferate under certain conditions, such as after partial hepatectomy or liver injury. Therefore, the mechanism of liver regeneration has been studied for many years, but with little success until recently (11).

In primary culture, mature hepatocytes do not grow at all under usual culture conditions, but in 1976 Richman *et al.* showed that additions of insulin and EGF to the medium induced DNA synthesis (32). This finding has been confirmed by many others including us (7, 10, 14, 16, 27, 39). Moreover, we noticed that at a high cell density of near confluency (10^5 cells/cm²), DNA synthesis induced by these two hormones was slight in 20 h-culture, but became appreciable in 40 h-culture (39). As shown in Fig. 2, the lag period required for start of DNA synthesis becomes shorter with decrease of cell density (27). This lag period apparently corresponds to the time required for the cells to proceed from the G_0 phase to the G_1 phase, in which they respond to insulin and EGF and move to the S phase. This time required for the G_0 - G_1 shift depends on cell density. Thus at low cell density (3×10^4 cells/cm²) 70–80% of the hepatocytes entered the S phase in the presence of two hormones (Fig. 3A, B) and 36 h after hormone addition they showed an increase in cell number (Fig. 3C, D). It is interesting that the two nuclei of many binuclear cells seen in adult liver also synthesize DNA synchronously (Fig. 3B). As a result of cell division many mitotic figures can be seen, as shown by phase contrast and fluorescent microscopies of the same cells (Fig. 4). Unlike cells in skin and intestinal epithelium, which proliferate from basement stem cells and then differentiate, mature hepatocytes themselves divide and increase.

These results indicate that the first point of regulation of hepatocyte growth is at the step between the G_0 and G_1 phases. This point is regulated by cell-cell contact. Most differentiated cells *in vivo* are supposed to be in the G_0 phase, and hence in studies on the mechanisms regulating growth of functional cells or their carcinogenesis, more attention should be paid to this G_0 - G_1 shift. Moreover, on the basis of these

Fig. 2. Density-dependent synthesis of DNA. Hepatocytes at different cell densities (circles, 3×10^4 /cm²; squares, 6×10^4 ; triangles, 12×10^4) were cultured and at 5 h (open symbols), 20 h (semiclosed symbols) and 40 h (closed symbols) insulin (10^{-7} M) and EGF (20 ng/ml) were added. [methyl-³H]Thymidine was added 2 h before assay of DNA synthesis.

Fig. 3. Increases of DNA synthesis (A, B) and cell number (C, D) of hepatocytes cultured with insulin and EGF at low cell density. Hepatocytes (3×10^4 cells/cm²) were cultured as described in Fig. 2. [methyl-³H]Thymidine incorporation was measured by radioautography 24 h after additions of insulin and EGF (B) or at this time without hormones (A). Phase-contrast microphotographs were also taken 36 h after hormone addition (D) or at this time without hormones (C). $\times 200$.

considerations, it is clear that established cell lines, which are not in the G₀ phase and usually stay in the G₁ phase even under resting conditions, are not suitable for studies on either normal or abnormal cell growth; primary cultures of mature hepatocytes are the most suitable for such studies.

2. *Specific requirement of collagen for hepatocyte proliferation.* During studies of suitable culture media for hepatocytes, we noticed that for cell survival Leibovitz L-15 medium (L-15) is better than Williams medium E (WE), which we had usually used. However, hepatocytes in L-15, unlike those in WE, did not synthesize DNA at all even in the presence of insulin and EGF. A survey of other media showed that there were two types of media: Ham F-12, McCoy 5A, Parker 199, RPMI 1640 and WE media were suitable for DNA synthesis, whereas Eagle's MEM, Dulbecco's modified MEM and L-15 were unsuitable for DNA synthesis (Fig. 5). L-15 does not contain four amino acids (Asp, Glu, Cys and Pro) and four metal ions (Cu²⁺, Fe²⁺, Mn²⁺, and Zn²⁺) present in WE. Addition of the four metal ions to L-15 did not promote DNA synthesis in hepatocytes, but of the amino acids that are deficient in

Fig. 4. Mitotic figures of hepatocytes cultured with insulin and EGF. Left: phase-contrast micrograph, right: fluoromicrograph with Hoechst 33258. Cells were treated with the two hormones for 24 h and TN-16 was added for 6 h before fixation. Arrows in both panels indicate the same mitotic cells.

L-15, Pro was found to provoke marked DNA synthesis in L-15 in the presence of insulin and EGF (26). The other media that did not support DNA synthesis were also found to lack Pro and when supplemented with Pro they did support DNA synthesis, addition of Pro at 30 mg/l being most effective (Table 1). Conversely omission of Pro only from WE resulted in loss of its ability to support DNA synthesis and readdition of Pro restored this ability. These results showed that Pro is essential for DNA synthesis in mature hepatocytes.

Then, what is the role of Pro in hepatocyte growth? Pro is a major constituent of collagen, some Pro being specifically hydroxylated in procollagen. Therefore, we examined the effects of inhibitors of collagen synthesis on DNA synthesis in hepatocytes. Addition of *cis*-4-hydroxy-proline (*cis*-OH-Pro) to WE or L-15 supplemented with Pro (30 mg/l) caused dose-dependent inhibition of DNA synthesis (Fig. 6). The inhibition was maximal with *cis*-OH-Pro at 120 μ g/ml, which did not show any cytotoxicity or inhibit protein synthesis. The inhibition by *cis*-OH-Pro was reversible, because addition of 5-fold excess of Pro to WE containing *cis*-OH-Pro resulted in recovery of ability to support DNA synthesis. Other inhibitors of collagen synthesis, such as α,α' -dipyridyl and D-penicillamine, which inhibit proline hydroxylase and maturation of collagen fiber, respectively, also inhibited DNA synthesis in hepatocytes dose-dependently. Conversely, ascorbic acid, a stimulator of collagen synthesis, increased DNA synthesis 1.5 times.

These results showed that collagen synthesis in hepatocytes is closely related to their DNA synthesis. It should be mentioned that these results were obtained on hepatocytes cultured on dishes coated with collagen from rat tail, which is type I collagen. Addition of types II and IV collagen to cells cultured in L-15 also did not induce DNA synthesis. These results suggest that a specific type of collagen or its

Fig. 5. Effects of various culture media on DNA synthesis. Hepatocytes (5×10^4 cells/cm²) were cultured in various media and DNA synthesis was measured as described in Fig. 2.

endogenous synthesis is necessary for hepatocyte DNA synthesis.

The mechanism how collagen is involved in hepatocyte DNA synthesis is still unknown. The effect of Pro on DNA synthesis is seen only in the presence of insulin and EGF: Pro alone does not stimulate DNA synthesis. We found that Pro was required to stimulate DNA synthesis in the first 12 h after insulin and EGF were

TABLE 1. EFFECT OF L-PROLINE ON DNA SYNTHESIS

Medium	DNA synthesis stimulated by insulin and EGF (dpm/mg protein $\times 10^{-4}$)	
	—Pro	+Pro (30 mg/l)
WE	23.5 $\pm$ 1.71	
L-15	3.2 $\pm$ 1.09	23.7 $\pm$ 2.92
Eagle's MEM	4.8 $\pm$ 0.64	16.3 $\pm$ 0.95
Dulbecco's MEM	4.0 $\pm$ 0.24	18.7 $\pm$ 1.90

Hepatocytes at low cell density were cultured in various media. Insulin (10^{-7} M) and EGF (20 ng/ml) were added to cultures 20 h after plating and [methyl-³H]thymidine was added 14 h later. DNA synthesis was determined after 34 h. Addition of serum or coating dishes with rat tail collagen did not affect DNA synthesis.

Fig. 6. Inhibitory effect of *cis*-OH-Pro on DNA synthesis. Hepatocytes were cultured with the inhibitor and DNA synthesis was measured as described in Table I. Open circles, WE; open squares, L-15 supplemented with L-Pro (30 mg/l); closed circles, WE supplemented with L-Pro (150 mg/l); closed squares, L-15 supplemented with L-Pro (180 mg/l).

added and addition of *cis*-OH-Pro after this period showed little effect on DNA synthesis (unpublished), suggesting that collagen is required in the early G₁ phase, but not in the transitional stage from the G₀ to G₁ phase.

Studies on growth of Balb/c 3T3 cells showed that first PDGF makes the cells competent and then insulin, somatomedin and EGF stimulate the cells to proceed to the S phase (31, 35). If a similar mechanism operates in hepatocytes, collagen may act as the factor making the cells competent, and then insulin and EGF may stimulate the hepatocytes to proceed to the S phase. However, regardless of the mechanism of its action, it is interesting that collagen, which was hitherto thought to be simply a component of connective tissue, plays an important physiological role in DNA synthesis. A similar idea was proposed by Enat *et al.* (5).

Hepatocytes in primary culture have been shown to synthesize collagen (4, 41), which is mainly type III with small amounts of types I and IV (4). Recently, electron microscopic studies on preparations treated with monoclonal antibodies against various types of collagen have shown that type I, III and IV collagens are present between hepatocytes and Disse's space (13). Therefore, hepatocytes *in situ* are in contact not only with each other, but also with the biomatrix composed of collagen and various glycoproteins, and various activities of hepatocytes must be influenced by this microenvironment.

In this section we have described the effect of collagen in the biomatrix; the effects of neighboring hepatocytes and a membrane regulatory factor are described in section III-1 and 2. These effects provide an understanding of the molecular mechanism of hepatocyte regulation as a liver tissue.

3. *Serum growth factor for hepatocytes (Hepatotropin, HTP)*. It has been postulated that active liver regeneration induced by partial hepatectomy is stimulated by increase in some growth factor(s) in the serum. This idea was suggested from findings in parabiotic rats (18) and experiments on liver transplantation (12). Since insulin and EGF stimulate growth of hepatocytes in primary culture, it has been thought that these two hormones may be the serum growth factor(s). There is no evidence, however, that the concentrations of these hormones in the serum increase after partial hepatectomy, and on the contrary, the level of insulin was shown to decrease after the operation. Moreover, the finding that the effective concentration of EGF for stimulation of DNA synthesis in hepatocytes in culture is 20–30 fold higher than that in serum adds further doubt to the idea that these hormones are serum growth factors.

Recent reports suggest the existence of a growth factor for hepatocytes in serum that is not EGF or PDGF (16, 36). We also found a serum factor that promotes DNA synthesis in hepatocytes in culture in the absence of insulin and EGF (23). The concentration of this factor increased in the serum of rats after partial hepatectomy. We isolated this factor by Sephadex G-200 chromatography, which showed that it had a molecular weight of 150 KD. This factor is acid labile and inactivated by heating at 90°C for 3 min and by trypsin treatment, indicating that it is an acid- and heat-labile protein. This serum factor had additive effects with insulin and EGF in stimulating DNA synthesis (Table 2), suggesting that their mechanisms of action are different. We named the serum factor hepatotropin (HTP) (23). In whole serum the effect of HTP was hard to observe, but it could be detected in a fraction of molecular weight 150 KD obtained by gel filtration. In normal rat serum low activity of HTP was detected, but after partial hepatectomy it increased 3–5 times. It increased appreciably 12 h after the operation, reaching a maximum after 24 h and then gradually decreasing. In attempts to purify HTP, we found that it was not adsorbed to CM-cellulose at neutral pH, but was adsorbed to DEAE cellulose, suggesting that it is an acidic protein. It could be purified 20-fold by heparin-Sepharose CL-6B column chromatography. Some possible growth factors, such as PDGF, FGF, somatomedins, thrombin and transferrin did not have any effect on

TABLE 2. EFFECTS OF HTP, INSULIN AND EGF ON DNA SYNTHESIS AND THE LABELING INDEX

Hormones	DNA synthesis (dpm/mg protein)	Labeling index (%)
None	420 ± 287	1.3 ± 2.2
Insulin (I, 10 ⁻⁷ M) + EGF (20 ng/ml)	9905 ± 810	29.6 ± 5.1
HTP-A (2.4 mg/ml)	8032 ± 268	27.9 ± 4.7
HTP-B (86 µg/ml)	5239 ± 309	15.1 ± 2.2
HTP-A + I + EGF	16885 ± 1879	52.3 ± 4.8
HTP-B + I + EGF	13883 ± 1031	44.9 ± 9.0

Hepatocytes were cultured in WE as described in Table 1, except that DNA synthesis was measured 24 h after addition of [methyl-³H]thymidine. Fraction HTP-A was obtained by Sephadex G-200 chromatography and HTP-B by heparin-Sepharose chromatography.

DNA synthesis, suggesting that HTP in rat serum is a new growth factor.

Recently, a hepatocyte growth factor that was not identical with PDGF was reported by Bucher's group to be localized in rat platelets (36). This factor was partially purified and found to be a basic, heat-labile protein with a molecular weight of 65 KD (33). Recently, Michalopoulos *et al.* reported that rat and human sera contain two growth factors for rat hepatocytes in culture (17). One has a molecular weight of over 120 KD and is heat-labile, while the other is less than 3 KD and is heat-stable. Morley and Kingdon also reported the presence of a hepatocyte growth factor in the serum of rats after partial hepatectomy (20). This factor has a molecular weight of 26 KD and is heat-stable. Thus there seem to be several hepatocyte growth factors in the serum. Comparative studies of these growth factors are necessary after their further purification. The origins of these factors are also of interest. Although we could not detect any HTP activity in extracts of platelets or regenerating liver, our recent work suggests that ATP is localized in platelet together with its inhibitor and that the two are secreted sequentially after partial hepatectomy (unpublished data).

III. Control of growth and functions of hepatocytes by cell-contact; cell sociological consideration

1. *Reciprocal modulation of hepatocyte growth and functions by cell-cell contact.* In liver lobules, hepatocytes are arranged radially between the peripheral portal vessels and central veins and are in contact with each other on one side and with the biomatrix on the other side. This liver microarchitecture with cell contact should control the hepatocytes, resulting in their expression of fully differentiated characters and suppressing their growth. In this *in vivo* state, the hepatocytes are in the G_0 phase and this state can be reproduced *in vitro* as described in section II-1. When hepatocytes are cultured at high cell density (confluency), which is about half that *in vivo*, their DNA synthesis is suppressed and their liver specific, differentiated functions are expressed maximally (Fig. 7 and Table 3). For example, the hormonal inductions of

Fig. 7. Reciprocal effects of hepatocyte density on growth-related and differentiated functions. Culture conditions were as described for Table 3.

TABLE 3. RECIPROCAL EFFECTS OF CELL DENSITY ON GROWTH RELATED AND HEPATOCYTE SPECIFIC FUNCTIONS IN PRIMARY CULTURES

Function	Stimulators	Activity (%)		
		Cell density ($\times 10^4$ cells/cm ²)		
		2.5	7.5	12.5
DNA synthesis	insulin+EGF	100	36	5
Amino acid transport	insulin	100	67	42
Protein synthesis		100	84	52
Cholesterogenesis		100	75	37
G6PD induction	insulin+EGF	100	38	23
Triglyceride synthesis	insulin	33	88	100
induction of				
ME	insulin+triiodothyronine	34	50	100
TAT	dexamethasone	11	47	100
TO	dexamethasone	13	55	100
SDH	dexamethasone+glucagon	13	44	100

Hepatocytes were cultured at various cell densities for several hours and then the indicated hormones were added and various cell activities were assayed 24–48 h later.

tyrosine aminotransferase (TAT), serine dehydratase (SDH) and tryptophan oxygenase (TO), which are related to gluconeogenesis, and hence are liver-specific functions, are maximal at this high cell density, although they are very low at low cell density. In contrast, activities that are related to growth, such as DNA synthesis, amino acid transport, protein synthesis, cholesterogenesis and induction of glucose-6-phosphate dehydrogenase (G6PD) are regulated in the opposite way by cell density; namely, they are high at low cell density and low at high cell density. It is interesting to find that of the lipogenesis, cholesterogenesis is regulated by cell growth, while synthesis of triglycerides is regulated by cell differentiation. This difference can be explained by supposing that cholesterogenesis is necessary for membrane biogenesis during cell growth, whereas triglyceride synthesis is accelerated for lipoprotein secretion in mature hepatocytes. Similar opposite regulations of the two enzymes producing NADPH, namely G6PD and malic enzyme (ME), were observed. G6PD may supply NADPH for cholesterogenesis and ribose-5-phosphate for nucleic acid synthesis during cell growth, while ME may supply NADPH and oxaloacetate for fatty acid synthesis in mature hepatocytes. These explanations are supported by the finding that EGF induces G6PD, but suppresses ME (42).

This regulation of hepatocyte differentiation is based on a transcriptional mech-

TABLE 4. EFFECT OF CELL DENSITY ON TRANSCRIPTION OF TO

Cell density ($\times 10^4$ /cm ²)	mRNA activity (In vitro translation)	Amount of mRNA (Dot hybridization)
3.1	0.031 ^a	18 ^b
6.2	0.118	117
12.5	0.222	214

Hepatocytes were cultured as described for Table 3. ^a % of total mRNA. ^b Arbitrary densitometric units.

anism like that for DNA synthesis; we showed that the regulations of SDH and TO by cell density are due to change in mRNA activities of these enzymes. Furthermore, change in the amount of TO mRNA in hepatocytes at low and high density was shown by Northern hybridization with TO cDNA (Table 4). Therefore, these reciprocal modulations of hepatocyte growth and differentiation by cell density are possibly controlled at the gene level.

2. *Cell surface modulator (CSM)*. From the findings mentioned above it is interesting to investigate the mechanism underlying this elaborate reciprocal regulation of growth and functions of hepatocytes. Two possibilities may be considered. One is an autocrine, humoral factor secreted by hepatocytes themselves and the other is a membrane-bound factor through cell-cell contact.

The first possibility was examined by adding conditioned medium from cultures at high cell density to cultures at low cell density and examining the latter for activities related to growth and differentiated functions mimicing those at high cell density. Results showed that conditioned medium had no effect.

The second possibility was examined by adding plasma membranes from liver to hepatocytes cultured at low cell density to see whether these membranes could mimic the effect of high cell density. As shown in Fig. 8, addition of highly purified plasma membranes from rat liver caused dose-dependent suppression of DNA synthesis and induction of TAT activity in hepatocytes cultured at low cell density (29). Addition of plasma membranes also resulted in change in shape of hepatocytes to resemble that of hepatocytes in dense cultures; that is, reduction in cell spreading and formation

Fig. 8. Effect of addition of hepatic plasma membranes to hepatocytes cultured at low cell density in stimulating TAT induction (circles) and suppressing DNA synthesis (triangles). The membranes were added to cultures 2 h after cell plating and various hormones were added 22 h after addition of membranes. TAT induction and DNA synthesis were measured 24 h after hormone addition. Open circles are values without addition of dexamethasone.

of small, round cells. The effect of the plasma membranes was inhibited by heating them (95°C for 2 min, or 65°C for 15 min) or treating them with trypsin (1 µg/ml for 15 min).

These results indicate that the effective factor in the membranes is a heat-labile protein. This factor could not be solubilized from plasma membranes of liver by treatment with ordinary detergents, but was solubilized by their treatment with 4% octylglucoside plus 4 M guanidine-HCl (21). This solubilized factor was found to be a protein of 670 KD by Sephacryl S-400 chromatography. When this factor was treated with trypsin, a specific fraction of 200 KD seen on SDS-polyacrylamide gel electrophoresis disappeared with concomitant loss of modulator activity. We named this active protein cell surface modulator (CSM). Since the stimulatory and suppressive activities on differentiated functions and DNA synthesis, respectively, could not be separated during purification or by various treatments, it is likely that they are properties of the same protein. CSM was found in plasma membranes of not only liver, but also kidney, brain and red cells. It is likely, therefore, that specific expression in each type of cells is determined by their genetic factors and SCM only modulates these expressions.

3. *Possible mechanism of regulation of liver regeneration by CSM and of hepatocarcinogenesis by loss of CSM response.* As described above, hepatocytes *in vivo* are in tight cell-cell contact in a lobule and CSM on their membranes must give a signal to suppress their growth and stimulate their expression of differentiated characters (Fig. 9). Therefore, the bifunctional character of CSM would be suitable to control specific expression of cells in tissue, in other words it could be called tissurization factor.

When cell-cell contact in liver is disturbed by an injury, such as in viral hepatitis, by partial hepatectomy or on treatment with hepatotoxin, suppression of growth by CSM would be lost and surviving hepatocytes in the G₀ phase would shift to the G₁ phase, where various humoral factors, such as insulin, EGF and HTP would stimulate the cells to move on to the S phase and divide. During this cell cycle, the biomatrix including collagen may be important for their smooth shift. When the hepatocytes have divided

Fig. 9. Possible mechanism of reciprocal regulations of cell growth and functions by SCM.

once or twice, then cell contact becomes tight again and through CSM regulation the cells go back to the G_0 phase, ceasing cell division and expressing specific functions. This is the normal mechanism for wound healing or liver regeneration.

This mechanism may apply not only to hepatocytes, but also to other cell types in liver, such as connective and endothelial cells. If the liver injury is continuous, growth of all cells, particularly fibroblastic cells, would also become continuous and they would become predominant in cell population of the tissue. This would explain the condition in liver cirrhosis due to chronic hepatitis.

The question arises of how this mechanism applies to liver regeneration after partial hepatectomy, in which the remnant tissue seems to retain intact and tight cell contact. But in fact contact of hepatocytes is actually loose, because gap junctions between cells disappear and intercellular communication, such as transfer of small molecules and electric coupling, decreases (15, 38). Recently, it was shown that connexon, a protein in gap junctions, disappears after partial hepatectomy with concomitant loss of electric coupling (40). It is also noteworthy that if the cells in the remnant of liver after 70% partial hepatectomy were to divide only once or twice, their cell number would become that of the original liver.

This control mechanism of growth and functions could also be applied to hepatocarcinogenesis. If the response of hepatocytes to CSM is lost, as discussed above, then they would stay in the cell cycle. In general, cancer cells show loss of intercellular recognition and tissue structure. We examined whether hepatoma cells have lost CSM or the response to CSM using the differentiated Morris and Reuber hepatoma cell lines in culture, H₄-II-E and MH₁C₁, respectively. These cells retain a few differentiated functions, such as showing induction of TAT by dexamethasone. However, these differentiated functions and DNA synthesis do not show any cell-density dependence, although the plasma membranes of the hepatoma cells retain CSM activity (22), because these membranes can reciprocally modulate DNA synthesis and TAT induction of normal hepatocytes cultured at low cell density. CSM was also found in other cancer cells, such as Morris 7794A, Ehrlich ascites tumor and p-388 leukemia cells. Therefore, loss of regulation by cell-cell contact in tumor cells is not due to loss of CSM, but possibly to loss of the response to CSM of these cells. Whether this loss of a response is due to loss of a CSM receptor or of post-receptor signal transmission is still unknown.

Here, we would like to propose a possible mechanism for the acquisition of autonomous growth and dedifferentiation during hepatocarcinogenesis as loss of CSM modulation at the plasma membrane level, as shown in Fig. 10. By viral infection or carcinogen poisoning, as discussed above, hepatocytes in the G_0 phase may enter the cell cycle and at the S phase, DNA could be modified resulting in loss of the response to CSM of the newly divided cells. In this way, cells are immortalized first and further cell division in a carcinogenic environment may cause more commitment of these cells to the carcinogenic state. Recently we induced preneoplastic nodules in rat liver by treatment with diethylnitrosamine and 2-acetylaminofluorene and found that in these lesions expression of TO was totally suppressed while that of G6PD was markedly increased (19). This finding supports the idea that precancerous parenchymal hepatocytes have lost the response to CSM and that their expression of differentiated markers is suppressed, while their proliferation is accelerated.

Recent studies revealed that some oncogenes are closely related with PDGF, EGF and their receptors. If HTP, CSM and their receptors described here are

Fig. 10. Possible mechanism of autonomous growth and dedifferentiation of cells during carcinogenesis with special reference to CSM.

related with known or unknown oncogenes, it would be more interesting to understand the mechanism of hepatocarcinogenesis.

IV. Concluding remarks

In the last ten years many workers have found that primary cultures of mature hepatocytes are the best system for studies on liver functions and hormonal regulation. In addition, recent studies have shown that this system is also very useful for elucidation of the mechanisms of liver regeneration, carcinogenesis, differentiation and tissue formation. These studies have indicated that not only various hormones, but also a membrane factor (CSM) and environmental factor (specific collagen) are very important for hepatocyte growth and functions. That is, hepatocytes do not live by themselves, but are under the control of hormones, neighboring hepatocytes and other cells and the biomatrix. Understanding of liver as an organized tissue, not only in histological, but also in biochemical terms is now just beginning.

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