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Membrane Biochemistry and Chemical Hepatocarcinogenesis

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ABSTRACT: Biochemical membrane alterations appearing during the process of chemical carcinogenesis are described. Emphasis is put on membrane composition, structure, and biogenesis. In this presentation the knowledge gained from experimental studies of liver and skin in the process of cancer development is acknowledged. Important biochemical changes have been reported in lipid composition, fatty acid saturation, constitutional enzyme expression, receptor turnover and oligomerization. Functional consequences of the altered membrane structure is discussed within the concepts of regulation of cell proliferation, regulation of membrane receptor expression, redox control, signal transduction, drug metabolism, and multidrug resistance. Data from malignant tumours and normal tissue are addressed to evaluate the importance of the alterations for the process and for the eventual malignant transformation.

KEY WORDS: liver nodules, hepatocarcinogenesis, biomembranes, growth regulation, drug resistance.

I. SCOPE OF THE REVIEW

In this presentation a number of biochemical membrane alterations associated with neoplastic development and preneoplasia are reviewed. We realize that the field is extensive, both with respect to the number of different preneoplasias, and the membrane alterations described. In an attempt to build up a conceptual understanding of the biological behavior of premalignant cells, we have chosen to emphasize studies on one of the most well-characterized carcinogenetic models, the rat liver model. Based on studies using this model, structural and functional consequences of biochemical membrane alterations are discussed. Extensive and important work performed by many investigators on other models, species, organs, and biochemical topics have, therefore, been restricted to give room for mechanistic argumentation on the topics selected. The main objective of this presentation is focused on growth regulation and the mechanisms involved in the maintenance of growth advantage and the escape of growth regulation characteristic of neoplastic development and malignant transformation.

A. The Liver as a Model for Cell Proliferation

Cancer is a disease of cellular growth regulation. To understand the mechanisms by which the organism coordinates cell growth and differentiation, and how imperfections in this intricate control system create cells that escape the rules of restricted cell proliferation, studies have to be performed in cell systems where growth can be directed and monitored experimentally. In this aspect, the liver offers a suitable model for several reasons. The liver responds sensibly and actively to environmental changes, including car-

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cinogens, with a multitude of adaptive processes devoted to reestablish steady-state conditions compatible with survival and continuous life.[1] The processes of adaptation involve alterations at molecular and subcellular levels, as well as at levels of cell and tissue organization and cell proliferation that can be monitored with a variety of techniques. The liver is, in spite of lobular heterogeneities, a reasonably homogeneous organ with, for most investigations, an abundance of accessible cells exhibiting common reaction patterns. Modern techniques allow studies on cell-cell interaction and the impact on the target cell of the surrounding biomatrix, adjacent parenchymal and nonparenchymal cells, as well as the functional demands on the target cell determined by the workload and the total mass of liver parenchymal cells. Cell-dependent alterations can by synchronized, as can be the initial steps of the carcinogenic process. Cell proliferation, both mitogen-initiated and -maintained, as well as reparative, regenerative growth and programmed cell death, or apoptosis, can be turned on and off in a controlled and synchronized order in the liver, permitting studies of the importance of DNA replication, cell proliferation, and cell death on cellular adaptation and cancer development. In Tables 1 and 2 some experimentally available "livers" and liver cell populations are listed to illustrate the maneuverability of the liver model in studies of cellular adaptation and the regulation of cell proliferation.

In studies of carcinogenesis *in vivo*, there are no adequate control tissues available that satisfy the criteria required for reference tissues and cells. Normal liver is usually not relevant as a reference for proliferating preneoplastic and neoplastic tissue, because it is not proliferating and is not treated with carcinogens. Regenerating liver is proliferating, but too vigorously, and has not been exposed to carcinogens. The best control tissue is actually found within the model itself, i.e., the perinodular cells surrounding the tumorigenic lesions. However, this tissue is technically difficult to recover in a pure form, and the preparation of surrounding tissue will invariably be contaminated by small nodules. In histochemical studies, on the other hand, the perinodular areas are the best possible control. In Table 3 some of the most commonly used control "livers" for studies of

TABLE 1a
The Liver as a Model for Studies of Cell Proliferation *In Vivo*

Liver tissue Process	Labeling Index %
Normal liver	0.1
Regenerative liver	84
Latency	
Synchrony	
Stop	
Mitogen stimulated liver	80
Hyperplasia	
Apoptosis	
Mitoinhibited liver	0.1
Mitoinhibited liver under regenerative pressure	0.2–5
Clonal adaptation	
Resistance	
Preneoplastic liver	4–8
Initiation	
Promotion	
Progression	
Neoplastic liver	Varies
Progression	
Transplanted liver	Varies
Cell interaction	
Humoral regulation	

Note: Thymidine pulse 48 h.

TABLE 1b
The Liver as a Model for Studies of Cell Proliferation *In Vitro*

Liver tissue Process	Labeling Index %
Normal liver cells	
on growth-permissive biomatrix	10–20
Spontaneous cell growth	
differentiating biomatrix	5
Biomatrix growth control	
Liver cells exposed to growth regulators	
Competence factors	5
Progression factors	50–80
Growth inhibitors	0–1
Preneoplastic liver cells	
Spontaneous growth	30–40
Mitogen-stimulated growth	50–60
Mitoinhibition (TGF-β)	0
Biomatrix interaction	?
Neoplastic liver cells	Varies

Note: Thymidine pulse 48 h.

TABLE 2
Control Tissue in the Liver Model

Liver tissue	Labeling index %
Normal liver	0.1
Regenerating liver	84
Surrounding liver	0.5–1
Mitoinhibited liver (2-AAF)	0.02
Mitoinhibited liver after partial hepatectomy	0.2–5

Note: Thymidine pulse 48 h.

TABLE 3
Growth Properties of Liver Nodules

- Elevated growth fraction *in vivo* and *in vitro*
- Normal response to regenerative growth stimulus, but insufficient ability to shut off regenerative growth influence *in vivo*
- Normal response to TGF-β mitoinhibition
- Increased apoptotic cell death
- Diploid divisional growth pattern (regenerative growth)
- Resistance to certain growth-inhibitory xenobiotics

growth regulation and signal transduction are listed.

B. Liver Models for Carcinogenic Processes

An increasing number of experimental models have been developed over the past decades to study different steps and events in the hepatocarcinogenic process.[2] The models are based on different hypothetical mechanisms of action and have been developed for both sexes. In some models the lesions develop rapidly, whereas in other models the processes are slow. Some models are synchronized and require multiple experimental undertakings, while other models develop after initiation, with no further efforts from the outside. The different models are therefore appropriate for different mechanistic questions, and a comparison of the processes involved can be fruitful. All models produce liver cancer and in all models intermediate focal prestages, liver nodules, are generated. Considering the huge differences between the various models, it is amazing to find that the prestages of the different models resemble each other in most aspects hitherto studied, with very few exceptions. Irrespective of the agents used in the protocols to produce neoplastic liver lesions, e.g., genotoxic compounds with or without promotors, nutrient imbalance, bile acids, etc., liver nodules exhibit a very similar phenotype and biological behavior.[1,3]

In this overview we refer to work mainly conducted on either the resistant hepatocyte model described by Solt and Farber[4] or the model using intermittent feeding of 2-acetylaminofluorene described, with minor variations, by several authors.[5–8] The former model is sequential and synchronized,[9] permitting studies of events participating in the initiation, selection/promotion, and progression toward malignant transformation. The importance of DNA replication and regenerative growth for fixation of DNA perturbations and initiation have been demonstrated using this model, as well as the development of resistant cells and the relevance of regenerative growth stimulation in the promotion of the carcinogenic process.[2,10] The resistant hepatocyte model is an initiation/promotion model based on the hypothesis that chemical carcinogens alter rare hepatocytes by the formation of DNA adducts. In these cells, where DNA replication takes place before DNA repair eliminates the adducts, permanent and inheritable genetic changes arise. Rare cells acquire resistance to the mitoinhibitory effect of carcinogens and cocarcinogens in this process of initiation. For this reason, they can selectively proliferate in the presence of a mitoinhibitor, such as 2-acetylaminofluorene, in combination with a regenerative growth stimulus, usually accomplished by either partial hepatectomy or necrogenic doses of carbon tetrachloride. During a very brief selection/promotion regimen, initiated cells are clonally expanded to form liver foci and liver nodules. After termination of selection/promotion, the process is self-progressing. Most lesions in this model redifferentiate back to the normal liver phenotype during a few months following promotion,[11] leaving only a minor fraction of the nodules remaining and growing in the liver. In these persistent nodules, the carcinogenic process progresses to the eventual malignant transformation.[12-14]

In the model of intermittent feeding of carcinogens, prolonged administration of a single agent is used both for initiation and promotion, and the different steps in the process cannot be separated, at least not in the early stages. Intermittent feeding of carcinogens is used to produce large precursor lesions in large numbers, i.e., persistant, proliferating liver nodules in the later stages of the carcinogenic process. Individual nodules weighing 0.5 g can be regularly harvested, and crops of 5 g from a single rat are not unusual, permitting biochemical studies, such as subcellular fractionation requiring huge amounts of material.[8,15,16] In both models the nodules can be recognized fresh and can be isolated for investigations using a multitude of different techniques.

As previously indicated, liver nodules produced by different models share phenotypic properties, with the resistant phenotype standing as a hallmark of the preneoplastic cell. This has been demonstrated with over 75 different chemical carcinogens, as well as in models where nutritional imbalance is induced by a choline/methionine-deficient diet or a diet supplemented with orotic acid.[1,3,17-23] In liver nodules promoted by peroxisome proliferators, modification of the expression of certain phase II drug-metabolizing enzymes characterizing the resistant phenotype has been reported.[24] In other species, such as mice, deviations from the resistant phenotype have also been noted, where again components of phase II drug metabolism seem to diverge from the general picture in the rat.[25-27] Although most of the reviewed data refer to models using 2-acetylaminofluorene, there are indications[2] that the phenomena observed in this model are general and are also relevant to other liver models, as well as to liver tumors in other species, including human, and also in tumors from other tissues, such as lung,[28] pancreas,[29,30] bladder,[31] breast,[32,33] skin epithelium,[34] melanocytes,[35,36] stomach,[37] colon,[38] kidney,[39] mouth squamous epithelium,[40] and squamous epithelium of the uterine cervical mucosa.[41]

It is logical to assume that phenotypic characteristics shared by several or all models are important for the development of the lesion and its biological fate. The commonality between different liver models makes it relevant to assemble data from different laboratories and to try to fit the information into a general hypothesis explaining the development of liver nodules and the subsequent malignant transformation. The most important phenomenon on which the review is focused is the growth advantage expressed by liver nodular cells from the time of promotion through the entire process.

C. The Concepts in Focus

In this presentation we discuss biochemical modifications appearing mainly in the biomembranes of liver nodules and that conceivably could contribute to our understanding of the emergence, maintenance, and discontinuous progress of liver nodules in the carcinogenic process. The prerequisites and favorable properties for growth advantage in promotion and growth advantage of persistent nodular cells are discussed. In this presentation five different areas are emphasized:

1. Alterations in membrane lipids and their role in membrane structure, stability, and function
2. Alterations in the products of the mevalonate pathway in neoplasia and the putative consequences for drug resistance and neoplastic cell proliferation
3. Changes in growth-factor receptor expression and its importance for growth regulation and the development of autonomous growth
4. Alterations in drug handling and the foundation of multidrug resistance as an adaptive response with importance for the carcinogenic process
5. Iron deficiency and its mechanisms and consequences for the growth of tumor precursors

II. THE RESISTANT HEPATOCYTE

A. Resistance as an Adaptive Phenomenon

In a recent commentary, Farber[1] summarized data from work in the field of carcinogenesis, especially hepatocarcinogenesis, and suggested that the onset of the carcinogenic process is adap-

tive in nature. The organism reacts to an unfriendly hostile environment with adaptive responses, including (1) acute transitory reactions affecting most cells and (2) clonal constitutive adaptation, with clonal development of new cell populations resistant to the noxious environment. The former type of reaction involves induction of drug-metabolizing enzymes, acute-reactive proteins, heat-shock proteins, and DNA repair enzymes. The latter adaptive response encompasses the appearance of rare, single cells, scattered throughout the organ, which acquire resistance and become able to grow in the presence of the noxious agent. This growth advantage is the fundamental prerequisite for the clonal expansion of the altered cell population and its survival in a cytotoxic environment. The clonal adaptation phenomenon is reversible in the sense that the phenotypic alterations in growth behavior and in structural and biochemical organization redifferentiate after termination of the cytotoxic exposure.[1,2,11,42] The adaptive nature of a genetically programmed, clonal response of accommodation to environmental stress in the liver is supported mainly by two observations. First, the appearance of nodules in the livers of rats treated with a multitude of carcinogenic protocols endows the rat with a survival value, with dramatically reduced death rates on doses of, for example, carbon tetrachloride, that are normally lethal.[43] Second, the complex biochemical phenotype of liver nodules can be reversibly induced, with a nonclonal response, by agents such as lead nitrate,[44] interferon,[45] butylated hydroxytoluene, and butylated hydroxyanisole.[46,47]

In the concept of clonal adaptation as the trigger of the carcinogenic process, neoplasia can be considered as a manifestation of imperfection of the adaptive process.[1,48] Aberrations at the genomic level generate cells which after clonal expansion persist and progress in a multistep, clonal process to malignant transformation. As stated previously, the phenotype of the resistant, clonal cell populations generated in a multitude of different animal models of hepatocarcinogenesis[2] is surprisingly constant. The list of representative properties comprises functional resistance to the mitoinhibitory effect of many carcinogens,[11,49,50] resistance to cellular degenerative changes seen in surrounding and normal cells,[51-55] structural rearrangements,[56,57] and biochemical alterations.[58-60] Interestingly other tissues as well, such as bladder,[31] skin,[34] colon,[38] pancreas,[29,30,61,62] breast,[32] and respiratory tract,[28] show focal lesions regarded as preneoplastic or premalignant, with properties in common with the liver nodules. Furthermore, an increasing number of human preneoplasias and carcinogens[37,39,41,63-66] share phenotypic properties with the rat liver models. In fact, human malignancies with primary or acquired multidrug resistance exhibit many of the resistance hallmarks included in the resistance phenotype of liver nodules.[35,67-69] Thus, the broad and unspecific resistance profile does not seem to be dependent on and unique for the regimen that generated the response. Furthermore, resistance develops against harmful formulas that the organism has never previously been exposed to or evolutionarily never could have possibly met.

The ubiquitous genetic program for cellular adaptation can be regarded as a complete and general escape concept. It protects the organism from hazardous exposure that is too strong, too complex, or too chronic to be coped with by drug-specific and drug-dependent, reversible induction of metabolism and excretion processes. It is of biological importance to clarify the individual protective mechanisms of the resistance phenotype in order to understand the mechanisms of the escape process, as well as the onset of cancer development. The mechanisms of genetic regulation of complex alterations involving multiple genes are not known. A better knowledge of what is turning the program on and off would considerably help in our efforts to understand the biological process of adaptation and development of disease. For the treatment of human malignancies, knowledge of the complexity of the resistance phenotype will help oncologists to design more efficient tumor treatment protocols, bypassing the insusceptibility for chemotherapeutics.

B. Resistance in Hepatocarcinogenesis

The clonal expansion of initiated cells requires cell proliferation. In carcinogenic protocols, focal proliferation takes place under circumstances in which normal cells do not grow. In fact, carcinogens are mitoinhibitors for normal hepatic and other cells.[1,49] For instance, pheno-

barbital, which is regarded as a mitogenic substance and an inhibitor of programmed cell death, or apoptosis,[70,71] was recently found to inhibit S-phase DNA synthesis and growth response in an *in vitro* cell proliferation assay (Sarma, personal communication). In addition, phenobarbital reduces EGF-receptor expression and affects the proliferative response of hepatocytes to growth-promoting calcium concentrations *in vivo*.[72] The results imply that phenobarbital also inhibits hepatocyte proliferation *in vivo*. In fact, Tatematsu et al.[73] showed that phenobarbital inhibited normal liver-cell proliferation and stimulated nodular cell proliferation in an *in vivo* rat liver model for hepatocarcinogenesis. Orotic acid, a precursor in nucleotide biosynthesis and a potent tumor promotor, is also a growth inhibitor in proliferating primary cultures of hepatocytes plated on growth-permitting biomatrix.[74,75] Using *in vivo* ^{3}H-thymidine labeling, it has been shown that cells forming liver foci and nodules proliferate during 2-acetylaminofluorene promotion, when the growth of surrounding cells is inhibited.[11] In female rats, this differential mitoinhibitory effect of 2-acetylaminofluorene is less pronounced, permitting noninitiated cells to respond to regenerative growth stimulus following 1 week of drug treatment.[76] Hence, in females foci grow more slowly, with fewer nodules and carcinomas developing and at later stages than in male rats, where a complete 2-AAF mitoinhibition of the surrounding cells is achieved.[77,78] These data and data from models in which a prolonged regenerative growth stimulus is created by other means, such as with lasiocarpine,[54] choline-methionine deficient diet,[79] etc., suggest a mechanism of differential toxicity and acquired resistance as an important and general mechanism for cancer development. As a further mechanism involved in the differential growth response of initiated vs. noninitiated cells, it was recently suggested that 2-acetylaminofluorene stimulated clonal proliferation by a selective mitogenic response with the induction of regenerative growth in initiated cells.[80] The evidence is indirect and further study of their model, based on 6 weeks of dietary administration of 2-AAF after initiation with 50 mg diethylnitrosamine/kg body weight and partial hepatectomy, is needed to state conclusively that 2-AAF has a direct growth-stimulatory effect that cannot be regarded as a regenerative response caused by differential cytotoxicity. However, 2-AAF exerts a downregulating effect on EGF-receptor mRNA expression and alters the binding affinity of the receptor.[81] A direct growth-stimulatory effect of 2-AAF would presumably involve activation of the EGF-receptor pathway, for example, by inducing the expression of hepatic TGF-α.

C. Growth Advantage in Neoplastic Development

In the autonomous stages of preneoplastic development, where promotion is not necessary to maintain a growth advantage, the fraction of proliferating cells is 50-fold higher in nodular tissue than in resting liver.[82] This population of cells responds to regenerative mitogenesis as efficiently as normal cells but does not reestablish the growth rate to the prestimulatory level, leading to an elevated growth fraction for a prolonged period of time. In addition, the rate of apoptotic cell death also increases proportional to the increased growth rate. These parameters determine the expansion of the cell population in the premalignant stage of the process.[71,82,83] *In vitro* studies using nodular cells in primary cell culture also show a higher than normal growth fraction.[84] Nodular cells *in vitro*, however, are similarly inhibited by the potent mitoinhibitor TGF-β as normal cells.[84] The growth pattern of liver nodules is of the divisional type seen in regenerative liver growth, and not of the polyploidizing type that predominates during normal liver growth and development, as well as in the growth response to mitogenic stimulus.[85]

The resistance of liver nodules is not only expressed as a resistance to mitoinhibition, but also as a resistance to degenerative alterations in the cells. For example, nodular cells do not die of necrogenic doses of carbon tetrachloride,[43] neither exhibit megalocytic degeneration after lasiocarpin exposure,[54] nor fatty degeneration after treatment with a choline-methionine deficient diet,[52] or upon exposure to orotic acid.[86] Furthermore, nodular cells do not respond with el-

evated lipid peroxidation and cell damage upon exposure to prooxidants like ADP-iron or ascorbate.[87]

D. Mechanisms of Resistance

It is obvious from studies of carcinogenesis and resistance, as well as from work dealing with multidrug resistance in malignant tumors, that multiple mechanisms are responsible for this broad and relatively unspecific resistance. It appears likely that a complex serenade of genes are simultaneously and persistently altering their expression when the resistant cells are formed. The cellular alterations involve different levels of organization and are seen structurally as well as functionally. The alterations are focal and not general, in contrast to adaptation caused by exposure to xenobiotics that results in enzyme induction. The focal clones have a different histological organization, with the formation of twin plates, acinus formation,[56] and a change in vascularization.[88-90] One consequence of this reconstruction of the tissue architecture is that the nodules are perfused more from the arteries than from the portal blood, and that the arterial fraction of the blood perfusing the nodules increases with increasing size of the liver nodules.[90] The significance of these architectural alterations with respect to resistance might be that toxic compounds from the environment, brought to the liver via the portal blood from the gut, preferentially reach the non-nodular cells, rendering the nodular cells less exposed.

Other changes demonstrated in resistant cells are biochemical in nature and are the topic of this review. Among them are noted considerable changes in the expression and regulation of drug-metabolizing enzymes; differences in lipid composition, lipid metabolism, receptor expression, and regulation; iron metabolism; and cell surface redox reactions, consistent with cellular resistance and growth advantage.

III. STRUCTURAL AND FUNCTIONAL ASPECTS OF MEMBRANE LIPIDS IN HEPATOCARCINOGENESIS

The composition of the lipid bilayer of biological membranes is of utmost importance for the properties of the membrane and consequently for the function of the cell. Membrane fluidity, stability, and permeability, as well as the kinetic and thermodynamic characteristics of many enzymes, are regulated by lipids.[91] The role of membrane lipid alterations in the biological behavior of tumor cells has been addressed by several laboratories, where analyses of a variety of tumors from different tissues using different analytical methods have generated data with great variations. Consistent lipid changes expressed in tumors and characteristic of the transformed state are not commonly found, and at the present time the relation between altered membrane lipids and neoplasia is not clear. However, in some hepatomas consistent elevations in cholesterol levels,[92-98] decreases in phospholipids,[98,99] and decreased concentrations of ubiquinone[93,94,99-105] and dolichol[93,94,102-105] have been noted.

The differences in the lipid composition of biological membranes in preneoplastic liver and hepatomas may have functional implications. The absolute and relative amounts of different phospholipid subclasses, as well as the type and position of phospholipid fatty acids, affect membrane structure, stability, and permeability, and influence many enzymatic functions.[52] Cholesterol, dolichol, and ubiquinone also regulate membrane structure and function. From the data available, the membrane lipid alterations of neoplastic cells would result in membranes with dramatic differences in physicochemical properties compared with normal, while the preneoplastic cells seem to compensate the functional consequences of changes in one lipid compartment with changes in another.[106]

It is evident from the available data that differences in membrane lipid composition between normal tissue and cancers are not necessarily reflected during the carcinogenic process and therefore are not noted in membranes from preneoplastic cells. In this presentation we will discuss some lipid alterations in precancerous and cancerous tissues that may have implications for cell proliferation and may affect the progress of the carcinogenic process, and eventually the malignant transformation. Alterations in the mevalonate pathway, including the regulation of cholesterol biosynthesis and its relation to cell proliferation, farnesylation of membrane pro-

teins, and ubiquinone modifications, are among the most intriguing findings discussed. In Tables 4–10 are summarized the lipid composition of preparations from normal liver tissue, preneoplastic liver nodules, and liver carcinomas.

A. Phospholipids

The total amounts of phospholipids, cholesterol, and triglycerides in membranes from rat liver nodules are not different from normal expressed as milligrams of phospholipid per milligram of protein or per gram of liver tissue (Table 4).[8,106] When the major classes of phospholipids in microsomal membranes from liver nodules are compared with those of normal microsomes, an elevation of phosphatidylinositol and a slight reduction in the relative amount of phosphatidylcholine has been observed (Table 5).[8] The fatty acid composition of the phospholipids in different nodular subcellular membrane fractions varies in different reports. The most consistent findings are reductions of 20:4 and 18:0[87,106,107] and increases in 22:6 and 16:0 (Table 6).[106,107] The pattern of fatty acyl moieties on carbon atoms 1 and 2 of the glycerol backbone of phospholipids is not different from the normal pattern.[106] In liver carcinomas from experimental rat hepatomas, a slight reduction of total phospholipids,[97,108-111] reduced phosphatidylcholine, and elevated levels of sphingomyelin[97] have been registered. Human hepatocellular carcinomas do not show significant deviations from normal in this respect.[94] Interestingly enough, both experimental and human hepatomas exhibit low levels of arachidonic acid in most phospholipid subclasses,[87,94,97] with a striking exception in plasma membranes from hepatoma 2788CTC, where the arachidonic acid content of phosphatidylinositol is reported to be 10-fold elevated.[97] Little consistency is found among other reported fatty acid alterations in phospholipids from hepatomas. Wood et al.[97] conclude in an extensive study that the unique fatty acid profile, specific for phospholipid subclasses and for membrane subfractions from normal tissue, is absent or much reduced in hepatomas. They also reported altered oleate/vaccenate ratios in membrane phospholipids from hepatomas, consistent with fluidity changes that may affect the insertion and transfer of integral membrane proteins and receptor-mediated membrane functions.

B. Phosphoinositides and Inositol Phosphates

Phosphoinositides and inositol phosphates are ubiquitous intermediates in the signal transduction from cell-surface receptors to effector systems in the interior of the cell.[112,113] Several growth-factor receptors mediate tyrosine phosphorylation and activation of the phosphodies-

TABLE 4
Lipid Composition of Liver, Liver Nodules, and Hepatocellular Carcinoma; Phospholipids, Cholesterol, and Triglycerides in Microsomal Membrane Fractions

	μg/mg of protein		
	Phospholipids	**Cholesterol**	**Triglycerides**
Rat			
Normal liver	360	36	24
Liver nodules	330	33	23
Hepatoma	↓	↑	
Human			
Hepatoma	↓	↑	

Note: For hepatoma trends in deviation from normal is indicated by arrows, due to difficulties in standardization of values from different laboratories.

Data from Reference 8, 99, and 106. See text for further details.

TABLE 5
Lipid Composition of Liver, Liver Nodules, and Hepatocellular Carcinoma; Relative Phospholipid Composition in Microsomal Membrane Fraction

	% of total phospholipids				
	PC	PI	PS	PE	Spm
Rat					
Normal liver	59	3.4	5.4	29	3.5
Liver nodules	53	8.4	6.1	29	3.6
Hepatoma	↓ (0.7)			↑ (2)	↑ (2)
Human					
Hepatoma	↑ (1.2)			↓ (0.86)	

Note: For hepatoma trends in deviation from normal liver is indicated by arrows, due to difficulties in standardization of values from different laboratories. Numbers in parenthesis indicate the relative content of phospholipids compared with normal.

Data from References 8, 99, and 106.

terase phospholipase C-gamma, which converts phosphatidyl-inositol-4,5-bisphosphate (PIP_2) into phosphatidyl-inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG).[112-114] Both products act as potent second messengers regulating calcium levels in the cell.[114] IP_3 can be further phosphorylated by a specific kinase to inositol 1,3,4,5-tetrakisphosphate (IP_4), which increases intracellular calcium levels, facilitating calcium uptake from the outside of the cell.[115] In liver nodules growth regulation is altered, as mentioned previously, and alterations in specificity and/or sensitivity of the signal transduction system in the cell might conceivably change the cellular response to hormone and growth-factor exposure. In liver nodules, the amount of phosphatidyl-inositol, the precursor for synthesis of the polyphosphorylated inositol compounds, is increased.[8] It is therefore of considerable interest to elucidate whether ligand-receptor controlled phosphatidyl-inositol metabolism has any mechanistic role in the development of autonomous growth during the carcinogenic process. Although the nodular phosphatidyl-inositol is elevated compared to normal levels, both phosphatidyl-inositol-4-phosphate and phosphatidyl-inositol-4,5-bisphosphate are normal.[116] After interaction with vasopressin as an activating ligand, IP_3 is rapidly formed by both cells (Table 7). In normal liver, a sharp IP_4 peak arises within a minute from ligand exposure. In liver nodules less IP_3 is formed, when measured per gram of tissue, and no IP_4 is formed.[116,117] The reduction of IP_3 formation corresponds to the downregulated receptor number. Preliminary results do not support the assumption that changes in IP_3 kinase or IP_3/IP_4 phosphatase can explain the absent IP_4 peak, as was shown for pancreatoma cells.[118] It is reasonable to assume that the altered IP_4 response in nodular cells affects nodular calcium homeostasis and possibly also growth regulation and the growth-factor response.

C. The Mevalonate Pathway

Contrary to the phospholipids, the amount of lipid products of the mevalonate pathway, mainly cholesterol, dolichol, and ubiquinone, are strikingly and consistently different in preneoplastic tissue, as well as in malignant tumors. As is evident from the results presented in Table 7–10, premalignant and malignant tissue, in fact, seem to be each other's antipodes. In liver nodules, cholesterol and cholesterylester levels are similar to normal liver[8] or are somewhat lower,[106] while the amount of dolichol is elevated,[100,106] together with a relative shift to longer polyiso-

TABLE 6
Relative Amounts of Arachidonic Acid of Membrane Phosphatidyl-Inositol (PI), Phosphatidyl-Inositol-4-Phosphate (PIP), and Phosphatidyl-Inositol-4,5-Bisphosphate (PIP_2) in Normal Liver, Liver Nodules, and Regenerating Liver from Rats

	% of total fatty acids					
	Arachidonic acid			Arachidonic acid/ palmitic acid ratio		
	PI	PIP	PIP_2	PI	PIP	PIP_2
Normal liver	36	11	8.0	4.9	0.40	0.21
Liver nodules	27	11	5.9	2.3	0.37	0.15
Regenerating liver	38	17	10	5.4	0.61	0.23

Data from Reference 108 and unpublished data.

prenes.[106] Ubiquinone is highly elevated in microsomes and lysosomes of liver nodules.[106] In carcinomas, cholesterol is increased,[92-98] while dolichol[93,94,102,103] and ubiquinone[92-94,100-104] are both strongly reduced. The length of the polyisoprene chains in the subclasses of dolichol is shorter[103] in human hepatomas. Most malignant tumors investigated, including hepatomas, exhibit elevated levels of cholesterol and sphingomyelin, increased saturation of fatty acids, decreased levels of dolichol, with an elongation of the polyisoprene chains and elevated levels of ubiquinone. The expected effects on the physicochemical properties of biomembranes with this altered lipid composition would be increased membrane stability and rigidity, with a decreased fluidity and reduced permeability. Furthermore, the changes in sphingomyelin content could increase the osmotic fragility[119] and would inhibit phospholipid exchange proteins[120] and phospholipases.[121]

Membranes from liver nodules exhibit small or no changes in total phospholipid and cholesterol levels. The increased saturation in phospholipid fatty acids may lead to an increased membrane stability, but increased membrane dolichol, particularly long polyisoprenes, and ubiquinone compensate for this effect by their influences on the physicochemical properties of the membranes.[106]

D. Regulation of the Mevalonate Pathway

One of the most consistent membrane biochemical alteration of tumor cells is their increase in cholesterol content.[94,122] The accumulation of cholesterol in tumor cell membranes, the activation of cholesterologenesis in proliferating cells, including tumor cells, and the deregulation of cholesterol metabolism in tumor cells have been thoroughly investigated since the discovery of the loss of cholesterol feedback inhibition on cholesterogenesis.[123] Cholesterol is required for cell growth and proliferation, and an increased rate of cholesterol synthesis is seen in proliferating cells and tissues.[123-125] Trentalance et al.[126] noted that in regenerating liver the activity of 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase increased early after partial hepatectomy, while cholesterol synthesis was elevated only after 16 h. It became evident that proliferating cells could satisfy their needs for cholesterol by increased *de novo* synthesis, as well as by increased uptake of exogeneous cholesterol. Goldstein and Brown[127] and Witte et al.[128] have shown that rapidly proliferating cells express elevated levels of cell-surface LDL receptors, which may facilitate cholesterol uptake.

Siperstein[124] suggested that the activation of HMG-CoA reductase in proliferating cells had

TABLE 7
Phosphatidyl-Inositol-4,5-Bisphosphate (PIP_2), Inositol-1,4,5-Trisphosphate (IP_3), and Inositol-1,3,4,5-Tetrakisphosphate (IP_4) in Cells from Normal Rat Liver and Rat Liver Nodules at Different Times after Addition of Vasopressin

Minutes after addition of vasopressin	% of level at 0 min			
	0	1	3	5
Normal liver				
PIP_2	100	80	55	20
IP_3	100	155	175	180
IP_4	100	590	490	90
Liver nodules				
PIP_2	100	95	70	60
IP_3	100	190	105	100
IP_4	100	95	102	110

Data from References 119, 120.

the purpose of not only producing cholesterol for the membranes of the growing cell, but also of initiating DNA synthesis via mevalonate metabolism by the formation of isopentenyl adenine and its hydroxylated derivative, zeatin.[129] The complexity of the regulation of the mevalonate pathway and its relation to growth and growth regulation has been outlined recently.[130] The mevalonate pathway and its regulation, as suggested by Goldstein and Brown,[130] with sterol isoprenoids, such as cholesterol, and nonsterol isoprenoids, such as dolichol, ubiquinone, and farnesyl, as end products, are shown in Figure 1.

The HMG-CoA reductase, the rate-limiting enzyme in the mevalonate pathway, is regulated at the transcriptional and posttranscriptional levels. In tissue-culture model systems it has been shown that the reductase enzyme protein can be increased 200-fold over the normal enzyme amount. The increase is due to an eightfold increase in mRNA production, a fivefold elevation of mRNA translation, and a fivefold prolongation of enzyme half-life.[131,132] The fine-tuned physiological regulation of the reductase requires both a sterol, like cholesterol, and a nonsterol metabolite synthesized from mevalonate. The sterol acts by repressing the SRE-1 (sterol regulatory element-1)[133] at the transcriptional level, while the posttranscriptional regulation is likely to be dependent on the production of isopentyladenin,[129] acting by the regulation of reductase translation[134] or mediated by farnesylated proteins.

The GTP-binding and GTP-hydrolyzing growth-regulating proteins $p21^{ras}$, encoded by *ras* protooncogenes and oncogenes, are translocated to the membrane and activated by covalent farnesylation of the cysteine four residues from the COOH terminus of the peptide. The amino acids on the C-terminal side of the farnesylated residue is cleaved and the cysteine is methylated on its COOH group.[135-138] After binding to the inner surface of the plasma membrane, the farnesylated *ras* proteins are further palmitoylated on two cysteines near the C-terminal end. These modifications render the proteins more hydrophobic and anchor them tightly to the membrane. Inhibition of mevalonate synthesis by HMG-CoA reductase inhibitors prevents the farnesylation of these proteins,[135,136] causes the accumulation of unfarnesylated forms of the proteins,[139] and stops growth.[140]

1. Cholesterol

Cholesterol regulates its own synthesis and the mevalonate pathway by feedback inhibition of the transcriptional activity of HMG-CoA synthase, the HMG-CoA reductase, and the LDL receptor genes. In tumor cells, cholesterol metabolism is deregulated and the feedback inhibition of HMG-CoA reductase is lost.[94,122] The defect in the cholesterol feedback system in malignant cells is not well characterized. The HMG-CoA reductase from tumor cells has structural, kinetic, and antigenic properties similar to the enzyme in normal cells,[141] but exhibit a higher activation state in tumor cells compared with normal, fetal, and regenerating cells.[142]

In contrast to non-neoplastic, regenerating cells, the LDL receptors[143,144] and chylomicrone receptor[145] are downregulated in tumor cells. It can be hypothesized that the deficient sterol uptake would account for the activated state of the HMG-CoA reductase and the synthesis of mevalonate necessary for the initiation of DNA synthesis. To validate this hypothesis, the apparent insensitivity of the HMG-CoA reductase to regu-

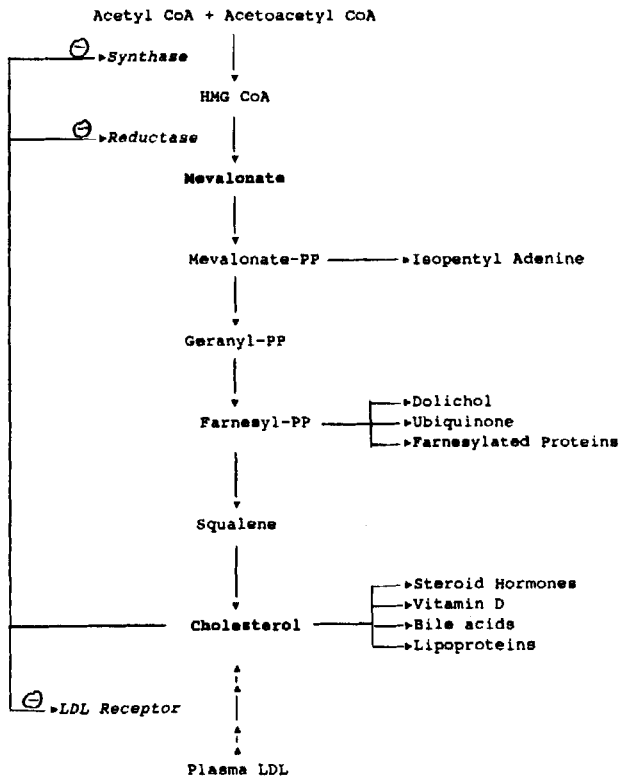

FIGURE 1. The mevalonate pathway in animal cells. Cholesterol is obtained from two sources: endogenously, by synthesis from acetyl-CoA through mevalonate, and exogenously from receptor-mediated uptake of plasma LDL. Homeostasis is achieved through sterol-mediated feedback repression of the genes for HMG-CoA synthase, HMG-CoA reductase, and the LDL receptor, as well as through post-transcriptional regulation of HMG-CoA reductase by non-sterol isoprenoids. (Data from Reference 130.)

lation by the accumulation of endogeneous cholesterol has to be explained, as well as the fact that tumor cells internalize and store exogeneous cholesterol.[123,146]

In liver nodules the products of the mevalonate pathway are altered in a different way than hepatomas. The cholesterol levels in the membranes are not altered or are slightly reduced,[8,106] while dolichol and ubiquinone are both elevated. The expression of LDL receptors is downregulated on the surfaces of cells prepared from liver nodules.[143] Ledda-Columbano et al.[147] showed an increased capacity for cholesterol synthesis in nodular tissue, as well as an enhancement of the pentose phosphate shunt associated with increased incorporation of tritiated thymidine into DNA.

The esterification of cholesterol in liver nodules is elevated, in contrast to dolichol esterification (see below), leading to a doubling of cholesteryl esters in different membrane preparations[106] when compared with normal cholesteryl ester amounts. The fatty acids found in cholesteryl esters of nodules are more saturated than those in normal liver. The functional consequences of increased cholesterol levels in liver cancer membranes may relate to changes in physicochemical properties, such as increased rigidity

and decreased fluidity and permeability.[148] Cholesterol has been shown to exert profound influences on cell-surface receptor characteristics and adenylate cyclase activity. Scarpace et al.[149] showed that preincubation of rat lung membranes with cholesterol hemisuccinate increased the number of β-adrenergic receptors and doubled the agonist affinity without changing the affinity for the antagonist. In spite of these receptor alterations, cholesterol incorporation decreased the catalytic activity of adenylate cyclase without affecting transduction of the hormone signal. Haeffner et al.[150] speculated that cholesterol asymmetry in the inner and outer lipid bilayer of plasma membranes may explain the stimulatory effect of cholesterol on the phosphatidylinositol-4-monophosphate kinase and phosphodiesterase. Membrane cholesterol also inhibits the activity of (Na^+,K^+)-ATPase by direct interaction with the enzyme protein.[151] Furthermore, Corwin et al.[152] showed that the transformed phenotype of the Kirsten sarcoma virus-transformed cell line, K3T3, was reversibly reverted when the cells were grown in delipidated fetal calf serum. This reversion could be blocked by the addition of cholesterol in combination with linoleic acid.

2. Dolichol

Dolichols are polyisoprenols with different chain lengths and are widely distributed in all eukaryotic biological membranes.[91,153,154] The phosphorylated form of dolichol has an established role in the biosynthesis of *N*-glycosidically linked oligosaccharide moieties of glycoproteins.[155,156] The functions of the esterified dolichols and the free alcohols are much less clear. It has been proposed that dolichols modify phospholipid fluidity, membrane stability, and permeability, and induce membrane fusion.[153,157,158] In rat liver nodules appearing during the carcinogenic process, dolichol levels are very high, with a fivefold increase in microsomal free dolichol compared with normal levels (Table 8).[106,159] A shift toward longer polyisoprenes is also noted (Table 9).[87,162] In contrast to dolichol, dolichyl phosphate and dolichyl esters are lower than nor-

TABLE 8
Lipid Composition of Liver, Liver Nodules, and Hepatocellular Carcinoma; Dolichol, Dolichyl-Ester, and Dolichyl-Phosphate in Homogenate and Microsomal Membrane Fractions from Rat and Human Liver Lesions

		ng/mg of protein	
	Dolichol	Dolichyl ester	Dolichyl phosphate
Rat			
Microsomes[106,162]			
Normal liver	72	18 (25%)	63 (17%)
Liver nodules	364		
Homogenate[162]			
Normal liver	201		36 (18%)
Liver nodules	565		23 (4%)
Human[106]			
Microsomes			
Normal liver	1390		130 (9%)
Hepatoma	890		250 (28%)
Homogenate			
Normal liver	3200	1820 (56%)	88 (3%)
Hepatoma	430	940 (218%)	91 (21%)

TABLE 9
Lipid Composition of Liver, Liver Nodules, and Hepatocellular Carcinoma; Distribution of Individual Dolichols Isolated from Homogenate and Microsomal Membrane Fractions from Rat and Human Liver Lesions

	Isoprenes (% of total)					
	D17	D18	D19	D20	D21	D22
Rat[87]						
Microsomes						
Normal liver	11	38	34	11	3.9	3.5
Liver nodules	7.1	30	37	17	6.5	13
Human[105]						
Homogenate						
Normal liver	2.8	12	38	33	12	2.5
Hepatoma						
Well differentiated	2.9	16	44	29	7.3	1.2
Poorly differentiated	13	16	42	24	3.8	1.2

mal in membranes from liver nodules (Table 8). Eggens et al.[159] showed that the reduction in dolichyl phosphate was related to a corresponding decrease in dolichol-mediated protein glycosylation, and it was suggested that the reduced glycosylation was due to a shortage of available dolichyl phosphate.

It has been proposed that a fatty acid moiety on dolichol serves as a targeting signal for the transport of dolichol from its site of synthesis in the microsomes to its final location in other membranes.[160,161] When esterification of dolichol is reduced, its transport to other organelles is accordingly retarded.[106,160] Furthermore, an increased level of saturation of the fatty acids of dolichyl esters might also impair the transport process. Elongation of the α-unsaturated isoprenoids may destabilize membrane structure.[153] It is possible that the reduction of dolichol esterification will inhibit transport of dolichols from the endoplasmic reticulum, resulting in an accumulation of the lipid and, due to a continuous condensation process, elongation of the polyisoprenoid chain.[106] In experimental rat hepatoma, a decrease in dolichyl phosphate has been observed[159] and dolichyl esters are low in experimental[106] as well as human liver cancer.[93,94] In spite of significant and contradictory differences in dolichol content and composition between preneoplastic tissue and cancer, the functional dolichyl derivates show more consistent alterations, indicating similar functional diversions from the normal cellular behavior.

3. Ubiquinone

Ubiquinone, or coenzyme Q, is a lipid-soluble compound widespread in animal and plant membranes[162,163] and in all lipoprotein subclasses.[164,165] Its main function is to serve as a redox component of the respiratory chain in mitochondria, but a second function, as a membrane and lipoprotein antioxidant, has been suggested for the reduced form, ubiquinol.[166] In hepatomas from rats[99,100] and humans,[94,103] the ubiquinone levels are decreased, while in rat liver nodules ubiquinone is elevated in microsomes and lysosomes, and is unchanged in mitochondria (Table 10).[106] A plausible functional implication of the increased ubiquinone levels in nodular membranes may be related to its effect as a potent antioxidant.[167,168] The total capacity of this antioxidant is inexhaustible and is dependent on the availability of reducing equivalents. The recycling of ubiquinol takes place in the mitochondrial respiratory chain, but electron-transport chains in microsomes[167,169] and plasma membranes[170] are also capable of restoring the reduced coenzyme. It is reasonable to suggest that the elevated levels of ubiquinone found in liver nodules contributes to the multidrug-resistant phenotype of the lesions due to its role as an antioxidant.

E. Lipid Peroxidation

Liver nodules have been shown to be resistant to necrogenic doses of CCl_4,[43] an agent known to induce rapid membrane lipid peroxidation and cell death. Benedetti et al.,[171] using a histochemical technique to detect protein-bound aldehydes (alkenals), showed that liver nodules did not accumulate lipid peroxidation products under conditions when the surrounding liver cells did. Poli et al.[87] found similar basal levels of lipid peroxides in liver nodules and normal liver, but in the presence of a prooxidant stimulus, like ADP-iron and ascorbate, liver nodules failed to in-

TABLE 10
Lipid Composition of Liver, Liver Nodules, and Hepatocellular Carcinoma; Ubiquinone Distribution in Homogenate and Membrane Subfractions from Rat and Human Liver Lesions

	ng/mg of protein				
	Uniquinone-9		Ubiquinone-10		
	Normal liver	Liver nodules	Normal liver	Hepatoma WD	Hepatoma PD
Rat[87]					
Microsomes	133	845			
Mitochondria	2365	2203			
Lysosomes	3418	8169			
Human[105]					
Homogenate			41	22	19

Note: Ubiquinone in rat has 9 isoprene units (Q-9) and in human 10 (Q-10).

crease lipid peroxidation. The resistance to CCl_4 and the low lipid peroxidation capacity in liver nodules may be explained by low levels of cytochrome P-450, low amounts of polyunsaturated fatty acids as substrates for lipid peroxidation, high glutathione peroxidase,[173] and glutathione S-transferase,[58] high amounts of glutathione,[3,173] and also the two- to sixfold increase in membrane ubiquinone.[106] Hepatomas and other malignant tumors, as well, are also resistant to oxidative stress and have lost their capacity to undergo lipid peroxidation[174-177] or to respond to prooxidants with increased lipid peroxidation.[87] This fact argues against the importance of ubiquinone as a protective agent against lipid peroxidation, since hepatomas are low in ubiquinone — 50% of control values. On the other hand, Burton et al.[178] and Cheeseman et al.[179] have found elevated levels of another lipid-soluble antioxidant, vitamin E, in tumors resistant to lipid peroxidation.

Lipid peroxidation has been proposed as a mechanism involved in initiation and promotion in the carcinogenic process. Rushmore et al.[180] noted nuclear membrane lipid peroxidation in livers of rats fed diets deficient in choline and low in methionine (CD diet) for only 1 d. After 3 d, liver DNA damage could be demonstrated.[181] They postulated that lipid peroxidation was responsible for the genetic perturbation in the initiation of the carcinogenic process after feeding CD diets.[182-185] Banni et al.,[186,187] however, claim that CD diets do not induce membrane lipid peroxidation. They state that lipid peroxidation products found in these animals on a CD diet are derived from lipid peroxidation of dietary lipids, mostly partially hydrogenated oils. The DNA lesions noted in the CD-diet-fed animals can be explained by a methyl group deficiency and single-strand breakage of DNA.[188] Increasing lipid peroxidation by a diet supplemented with polyunsaturated fat (corn oil) dramatically increased tumor yield, whereas hydrogenated lipids reduced tumor yield to less than half of that obtained with CD alone.[190] CD promotion induces hepatocellular carcinomas in rats[182-185] and mice,[191] and rapidly induces DNA damage[181] and regenerative cell proliferation.[192,193] Perera et al.[189] demonstrated a progressive insulin receptor downregulation and increased binding affinity during the administration of CD diets and suggested that these changes were related to the mechanism of CD tumor promotion. The disturbances caused by choline deficiency are seen mostly in the cells surrounding the liver foci and nodules, with a slightly increased cell death of noninitiated cells, while nodular cells are more resistant to dietary influences, lipid peroxidation, and cell death. The regenerative stimulus generated to repair the damaged liver preferentially stimulates growth of the initiated, resistant cells.

F. Lipid Alterations and Cellular Growth Advantage

The impact of the altered lipid phenotype on the increased growth rate found in these clonal liver lesions is not yet easy to envisage, although an activation of mevalonate synthesis due to impaired inhibition of HMG-CoA reductase by cholesterol is certainly an attractive theory. Coni et al.[194] recently found that the gene for HMG-CoA reductase is hypomethylated and overexpressed in liver nodules. This finding supports the idea that the gene is constitutively activated, rendering the mevalonate pathway resistant to regulation by normal feedback systems. The hypothesis is consistent with the finding that the reduction of serum LDL promotes cell proliferation and cancer development.[195] An increased channeling of metabolites from farnesyl-pyrophosphate into the polyisoprenol pathway, combined with low substrate affinity of squalene synthetase, could explain the altered lipid composition of the preneoplastic tissue. This alteration, in combination with elevated levels of NADPH due to increased activity of the pentose phosphate shunt, would warrant that mevalonate products and NADPH are not rate limiting for cell proliferation during the carcinogenic process.

IV. STRUCTURAL AND FUNCTIONAL ASPECTS OF MEMBRANE PROTEINS

A. Membrane Receptors During Chemical Hepatocarcinogenesis

Altered regulation of cell proliferation and differentiation are properties intimately associated with the malignant phenotype. These particular functional states of normal cell behavior are to a large extent regulated by the extracellular environment, such as by cell-cell and/or cell-matrix interactions, and by a multitude of humoral and tissue-specific soluble factors. These diverse regulatory factors share in common that their influence on target cells is initiated by binding to highly specific cell-surface proteins, e.g., binding proteins or receptors, which either transmit the information of the bound molecules over membranes into useful intracellular signals or mediate the uptake, or endocytosis, of extracellular molecules that are subsequently utilized in cellular metabolism.

There are three main components of cellular receptor systems that regulate cellular responsiveness. First, the extracellular ligand, which tightly and specifically binds to the extracellular domain of its cell-surface receptor; second, the membrane receptor itself, often activated by binding of the ligand; and, finally, the intracellular messenger system, which may be stimulated or repressed by receptor activation.

What membrane receptor alterations occur during carcinogenesis, and what are the molecular mechanisms underlying these receptor aberrations? Are similar changes observed during regulated cell proliferation? How is cellular responsiveness affected by the receptor alterations? Is the promotion of cancer cell development dependent on, or merely associated with, alterations of cell-surface receptor function? In this part of the review we attempt to discuss these and related questions by summarizing and comparing data on the structure and regulation of the EGF/TGF-α-, GH-, ASGP-, and transferrin receptors during liver regeneration as a model for regulated liver growth; in liver nodules containing preneoplastic cells formed during chemical hepatocarcinogenesis; and, finally, in the end product, hepatomas, developed from the liver nodules.

1. The Epidermal Growth Factor Receptor during Hepatic Regeneration and Hepatocarcinogenesis

Growth factors are a diverse group of polypeptides that regulate cell proliferation. Many growth factors operate in a paracrine, or in some cases an autocrine, fashion. This is in contrast to the action of classic endocrine polypeptide hormones, where the site of synthesis and the site of action often occur in different tissues or anatomical regions. One of the first growth factors to be discovered was epidermal growth factor (EGF), a 53 amino acid polypeptide with a molecular weight of 6045.[196] EGF stimulates replicative DNA synthesis in cells that express a specific cell-surface receptor, the EGF-R. The human EGF-R of M_r 170,000 to 180,000 is composed of a single polypeptide chain of 1186 amino acid residues and a substantial amount of *N*-linked

oligosaccharides.[196] A single hydrophobic membrane anchor sequence separates an extracellular ligand-binding domain from a cytoplasmic domain that encodes an EGF-regulated tyrosine kinase.[196] Since the cytoplasmic tyrosine kinase domain of the EGF-R is similar to a number of oncogene products, it is not surprising that the tyrosine kinase function of the EGF-R seems to play a dominant role in the regulation of cell proliferation.[197] The mechanism by which the binding of ligand to the extracellular part of the EGF-R activates its tyrosine kinase appears to involve an EGF-induced homodimerization coupled to intermolecular autophosphorylation of carboxy-terminal tyrosine residues.[197] In most cells expressing the EGF-R, the receptor exists in two states with respect to binding affinity for the ligand, where the minor fraction exhibits a binding affinity about one order of magnitude higher compared with the major fraction.[198] So far, no structural differences between these kinetically distinct receptor subtypes have been established, even though certain evidence suggests that the dimeric form constitutes the "high"-affinity form and the receptor monomer the "low"-affinity binder.[199,200] Conflicting data have, however, hampered any clear-cut conclusion regarding the involvement of "high"- and/or "low"-affinity EGF-R in the cellular actions of EGF, particularly the mitogenic effect.[199-201]

Shortly following ligand binding, the occupied EGF-R dimers are clustered into coated-pit domains of the cell surface, where internalization of the ligand-receptor complexes into intracellular endocytic vesicles is initiated.[197,202] The role of ligand-regulated endocytosis of the EGF-R is still unclear. Hypotheses regarding the functional role of this process range from attenuation of the biological response to the requirement of internalized receptor-ligand complexes to mediate some (or all) subsequent cellular responses to EGF.[202-204] What appears clear, as evident from *in vitro* mutagenesis studies, is that tyrosine kinase-negative EGF-R generates no cellular responses upon ligand binding[197] and that these mutated receptors are not degraded intracellularly following endocytosis, but are instead rapidly recycled back to the surface.[197,202] Thus, tyrosine kinase activity has been implicated in the sorting of internalized EGF-R for degradation. As a functional consequence of this kinase-dependent sorting, it could be predicted that ligand-induced downregulation would cause a relative enrichment of tyrosine kinase-defective receptors at the cell surface. Indeed, it has been demonstrated recently that induction of EGF-receptor downregulation is associated with a decreased formation of ligand-induced second messengers in hepatoma cells.[203] However, other studies have demonstrated that a noninternalizing mutated variant of EGF-R elicits an even stronger mitogenic response following ligand binding compared with wild-type receptors.[204]

In addition to EGF, there is a family of EGF-like molecules encoded by distinct genes.[197] Of these, transforming growth factor-α (TGF-α) is the best characterized candidate, fulfilling the function as the endogenous ligand for the EGF-R in most mammalian tissues, except possibly for epithelial cells lining the gastrointestinal tract.[205] TGF-α is synthesized locally in tissues and tumors as a glycosylated membrane-bound proTGF-α of 160 amino acid residues.[206] While most cells secrete the mature 50 amino acid residue form of TGF-α, which in most respects exerts the same actions at comparative potencies as EGF itself,[206] it is interesting to note that uncleaved proTGF-α can exert a mitogenic effect by activating EGF-R present on neighboring cells.[206]

In the adult liver, the rate of proliferation is low, i.e., only 2 out of 10,000 cells are dividing at any given moment.[207] However, during compensatory liver growth, such as seen after partial hepatectomy, where up to two thirds of the liver mass is resected, a synchronous wave of parenchymal cell replication is first noted at around 30 h after the surgery, when approximately 80% of the remaining cells are replicating.[207] Compensatory liver cell proliferation occurs after toxic and viral destruction of liver tissue, as well as during the initial stages of chemical hepatocarcinogenesis.[208,209] The factors that control initiation, progression, and termination of the proliferative response are now being rapidly defined, due to a considerable and renewed interest in the mechanisms underlying regulated liver growth.

EGF stimulates replicative DNA synthesis in mammalian hepatocytes in culture and *in vivo*,[207,210] suggesting a physiological role for EGF or EGF-like factors in liver cell proliferation. Expression of EGF-R are confined to parenchy-

mal liver cells,[211] where the number of EGF-R are in the range of 200,000 to 400,000 per cell.[211] In addition to EGF, a number of factors, such as insulin, norepinephrine, vasopressin, angiotensin, and the recently discovered hepatocyte growth factor (HGF), are potent liver-cell mitogens, often acting synergistically with EGF.[208,209] Regarding the expression of the EGF-R during the prereplicative phase of liver regeneration in the rat, the receptor synthesis and content of the cells in the liver remnant is transiently increased 1.5- to 2-fold, with a peak at 2 to 4 h after the operation.[212] This is followed by a significant downregulation, with the EGF-R mRNA levels reaching a nadir at around 50% 12 to 24 h postop, and the numbers of EGF receptors leveling out at an even lower value (Tables 10 and 11).[210,213-217] Interestingly, this decrease in receptor number appears to affect both "high"- and "low"-affinity receptors in cytoplasmic membranes to the same extent (Table 12).[217] Also isolated nuclei show an approximately 60% reduction of EGF binding sites at 8 h posthepatectomy.[218]

The levels of EGF-R are returned back to presurgery levels much later, at 72 to 96 h, coinciding with the cessation of cell proliferation.[210,213] The modulation of EGF-dependent tyrosine autophosphorylation of the EGF-R takes a similar course as the binding capacity and the receptor mRNA levels,[219] suggesting that the reduction in receptor number is mainly caused by decreased receptor synthesis.

Curiously, as is evident from studies on isolated hepatocytes in primary culture, cells harvested at 4 to 24 h after partial hepatectomy exhibit a marked increase in EGF-dependent stimulation of DNA synthesis with a peak at 24 h, despite very low numbers of EGF binding sites at the cell surface.[214]

The pattern of downregulation of EGF-R accompanying a proliferative state appears to also be conserved in liver nodules and hepatoma (Tables 11 and 12).[81,143,220,221] It thus appears that the quiescent hepatocyte express significantly higher levels of EGF-R than its proliferating counterpart. Although speculative, it is possible that the observed downregulation of the EGF-R is required for the initiation and/or progression of the proliferative event. What molecular mechanisms are responsible for the deregulated EGF-R expression in proliferating liver cells? With no definite answer at hand, one important factor could be activation of protein kinase C (PKC). Recently PKC was demonstrated to be capable of reducing the numbers of both "high"- and "low"-affinity EGF-R,[222] in addition to reducing the binding affinity of the high-affinity form of the EGF-R around 10-fold by phosphorylation of Thr-654.[223] It is presently unknown whether PKC regulates the oligomeric state of the EGF-R.

Yet another possibility comes from the recent demonstration of activation of autocrine TGF-α expression in regenerating liver, with a time course roughly similar to the downregulation of EGF-R.[224] Stimulation of downregulation and degradation of the EGF-R by EGF appears to require >50% saturation of cell-surface receptor sites, with less saturation allowing the receptor-ligand complex to rapidly recycle back to the cell-surface following internalization.[225] However, it remains to be experimentally verified that high levels of TGF-α are also capable of downregulating the hepatic EGF-R. This precaution is validated by the fact that in different cell types EGF, but not TGF-α, or vice versa, is capable of downregulating its cognate receptor.[226,227]

One approach to the study of the carcinogen-dependent changes in the expression of the EGF-R is to monitor the acute and long-term effects of different hepatocarcinogens. The potent hepatic tumor initiator and mutagen diethylnitrosamine causes, following a single necrogenic dose (200 mg/kg ip), a rapid decrease in hepatic EGF binding and receptor autophosphorylation, which is returned to normal values after around 30 d.[228] A similar but less rapid effect is seen when the very toxic herbicide and mutagen TCCD is ad-

TABLE 11
Quantitation of EGF-R mRNA Levels in Regenerating Liver, Liver Nodules, and Hepatoma

	EGF-R mRNA (amol/μg DNA)
Control liver	5.0 ± 0.45 (100)
Regenerating liver (18 h)	2.2 ± 0.50 (45)
Liver nodules	3.2 ± 0.30 (64)
Hepatoma	0.4 ± 0.20 (8)

Note: The EGF-receptor mRNA was quantitated by a solution hybridization method.[81,215]

TABLE 12
Number of EGF Binding Sites and Dissociation Constants in a Golgi Endosome Fraction from Regenerating Liver and Liver Nodules

	High-affinity sites		Low-affinity sites	
	N_0	K_D	N_0	K_D
Control liver	800 (100)	1.2	3,400 (100)	16
Regenerating liver (18 h)	110 (14)	1.8	540 (16)	12
Liver nodules	450 (56)	1.2	4,100 (120)	11

Note: A low-density membrane fraction containing Golgi and early endosome membranes was isolated as described in Reference 15. The number of EGF binding sites (N_0, fmol/mg protein) and dissociation constants (K_D,n*M*) were derived from Scatchard plots.

Data from References 81 and 217.

ministered as a single injection.[229] Regarding tumor promotors, regimens of choline-deficient (CD) or phenobarbital-supplemented (PB) diet fed to rats result in a prominent decrease in hepatocyte cell-surface EGF-receptor number at 10 d after the start of feeding.[72,230-232] In the case of PB, this reduction does not appear to involve PKC.[230] With the hepatocarcinogen 2-AAF, which has a strong promoting activity,[2,4] EGF binding is also decreased,[81,233,234] mainly due to a reduction in receptor number (Table 12).[233,234] Appearing as an exception to the rule, the only promotor substance that does not downregulate EGF receptors is ethinyl estradiol, causing a twofold increase, presumably due to stabilization of the receptor protein.[235,236]

In persistent liver nodules, resulting from a regimen of intermittent exposure to dietary 2-AAF for a total of 25 weeks, an interesting phenomenon occurs in which a reduction of high-affinity EGF-R is associated with a concomitant increase in the number of low-affinity receptors (Table 12).[81] The EGF-R mRNA levels are reduced to a similar extent as the high-affinity receptors (Table 12),[81] suggesting that the low-affinity receptors have a considerably longer half-life compared with the high-affinity receptors. Based on evidence presented previously, these low-affinity receptors may represent monomeric, noninternalizing cell-surface receptors. Although this hypothesis may seem plausible, particularly in light of a putative defect in the oligomerization of the asialoglycoprotein receptor in nodules,[237] it remains, however, to be experimentally established whether nodular cells have a diminished capacity for EGF-receptor dimerization and intermolecular phosphorylation. However, the potential importance of the low-affinity EGF-R for the mitogenic response to EGF has been addressed elegantly by Wollenberg et al.,[201] in which the EGF-dependent stimulation of DNA synthesis in primary hepatocyte cultures was found to be temporally associated with the transition from "high"- to "low"-affinity EGF-R.

Cells forming hepatocellular carcinomas developing as a result of chemical carcinogenesis in the rat characteristically express very low numbers of EGF-R, measured either as ligand binding[81,220,238] or EGF-R mRNA (Table 11),[81,221] compared with normal adult liver. The mechanism underlying this reduction of receptor number is therefore mainly exerted at the pretranslational level. In contrast, rat liver epithelial cells chemically transformed in culture appear to overexpress EGF-R, in addition to synthesizing TGF-α.[239-241] This contrasting phenotypic expression may indicate that paracrine factors derived from the surrounding nontumor tissue and/or systemic factors may act to maintain the expression of the EGF-R in malignant cells of hepatomas at a low level.

In the proliferating parenchymal liver cell of either normal, premalignant or malignant phenotype, a general finding is a low level of EGF-

R expression and, at least in some cases, an increased local production of TGF-α. The importance of TGF-α in the carcinogenic process has been demonstrated elegantly in transgenic mice overexpressing TGF-α, who frequently were found to develop hepatocellular carcinomas expressing elevated levels of TGF-α.[242] Unfortunately, no information regarding the corresponding levels of EGF-R was reported.[242] In light of the low EGF-R levels in neoplastic liver tissue, a lower sensitivity of malignant cells to EGF/TGF-α would be expected. This prediction is supported by a recent study showing that nodular and hepatoma cells *in vitro* have substantially reduced abilities to proliferate in response to EGF.[84] This property could eventually be compensated by high-level endogenous expression of TGF-α.

A possible role for (proto)oncogenes in the regulation of the EGF-receptor system during liver regeneration and hepatic carcinogenesis is suggested by recent studies, where transformation of mouse mammary epithelial cells by the introduction and induction of a point-mutated (and activated) *c-Ha-ras* protooncogene[243] or the *v-Ha-ras* oncogene,[244,245] was found to be associated with a concomitant downregulation of EGF receptor numbers and increased TGF-α production. Interestingly, the number of EGF receptors was inversely proportional to the amount of TGF-α secreted,[243] suggesting a causative relationship between these two parameters. Mutational activation of the *c-Has-ras* gene is, however, infrequently observed in carcinogen-induced rat liver tumors,[246,247] whereas increased expression of the *c-Ha-ras* and *Ki-ras* protooncogenes occur during liver regeneration,[248,249] coinciding in time with maximum downregulation of the EGF receptor (see above) and during chemical hepatocarcinogenesis.[250] It may be surmised that overexpression of *ras*-encoded proteins may play important roles, both in the altered growth regulation of preneoplastic liver nodules, as well as in the process of compensatory liver growth.

2. The Growth Hormone Receptor During Hepatic Regeneration and Hepatocarcinogenesis

Human growth hormone (hGH) is a 191 amino acid polypeptide with a molecular weight of 22 kDa. The relationship between GH and somatic growth has been known for a long time, although still very little is known about the cellular mechanisms by which GH exerts its growth-promoting effect. Since GH is not a particularly strong mitogen itself, research on GH-induced mitogenic factors was established in 1972 when the somatomedin hypothesis was launched.[251] The somatomedins, today referred to as insulin-like growth factors (IGFs) due to their structural similarity to insulin, were found to be mitogenic for cells, mainly mesenchymal, derived from a large variety of tissues. However, only one of the somatomedins, IGF-I, is regulated by GH.[252] It has recently been shown, by the use of the powerful transgenic mice technology, that IGF-I can sustain, in the absence of GH, normal growth of all body tissues except for the liver.[253] A second somatomedin, IGF-II, is highly expressed during fetal life,[254] when GH is absent, and has recently been found to be essential for normal fetal growth.[255] Thus, since IGF-I alone is not sufficient for promoting liver growth, this effect is presumably mediated by a direct effect of GH on liver cells.

In the rat, GH receptors are confined to parenchymal liver cells.[256] The hepatic GH receptors exist in multiple sized forms, with a larger variant of 95 to 100 kDa and a smaller form of 43 kDa, with the smaller form being the predominant species.[257] Occasionally an intermediate 52 kDa variant can be observed, probably a proteolytic fragment of the large GH receptor. The large and small receptor forms are encoded by distinct mRNA transcripts of 3.9 and 1.2 kb, respectively.[258,259] Analysis of the cDNA suggests that the small 43 kDa form represents a truncated variant of the large receptor form, generated by alterative splicing of the primary transcript,[260,261] and giving rise to a serum GH binding protein, produced in large quantities by liver parenchymal cells.[259]

Based on certain conserved homologies in the ligand-binding extracellular domain, it has been proposed that the GH receptor belongs to a hematopoetin receptor superfamily.[262] However, as to the signal transduction from the GH receptor, no significant similarities in the intracellular domain to other growth-promoting receptors can be deduced from the receptor sequence alone.[263]

GH binding to the large receptor form results in tyrosine phosphorylation,[264,265] suggesting that the liganded receptor is a substrate for tyrosine kinases present in the membrane. GH induce the protooncogenes *c-myc*[266,267] and *c-fos*,[267,268] and stimulate the turnover of phosphoinositides[269] and/or diacylglycerol.[268,270] These effects appear to involve activation of phospholipase C.[268,269]

One interesting molecular target for the GH receptor, presumably involved in the regulation of hepatocyte growth, is the receptor for epidermal growth factor. We[271] and others[272,273] have demonstrated that hypophysectomized rats and mutant dwarf mice, deprived of circulating GH, express very low levels of EGF-R, which can be restored by the administration of GH. This regulation is exerted mainly at the pretranslational level, as both EGF-R mRNA levels and EGF binding are affected in parallel.[271,272] Since GH itself is not mitogenic for hepatocytes in culture,[274] we speculate that the growth-promoting effects of GH on the liver are mediated by maintaining appropriate levels of EGF-R. In support of this notion, an excellent correlation between GH-R and EGF-R mRNA levels in regenerating liver, liver nodules, and hepatoma has been noted (Tables 11 and 13).[259,275]

In addition to its effects on IGF-I production and EGF-R synthesis, GH is responsible for maintaining a sexually differentiated hepatic metabolism of certain steroids and xenobiotics.[276] It is believed that the sex-different secretory pattern of GH, with a more continuous secretion in the female in contrast to the regular intermittent surges of GH secretion in the male,[277] acts by inducing and/or repressing specific key enzymes involved in sex-specific steroid and drug metabolism.[276]

The involvement of GH in the promotion of chemical hepatocarcinogenesis was initially hypothesized due to the higher incidence of liver carcinomas in males compared to females in both humans and rodents.[78] Thus, it was subsequently demonstrated that implantation of pituitary grafts under the kidney capsule to male rats after initiation with DEN, but before selection with 2-AAF/partial hepatectomy (Solt-Farber or resistant hepatocyte model), decreased the area, but not the number, of hyperplastic foci to the level of untreated female rats,[78] and also reduced the incidence of hepatoma formation in male rats.[78] Later experiments clearly showed that GH was the pituitary factor responsible for this effect following continuous administration.[278] Recently, nodules generated in the resistant hepatocyte model from male and female rats were found to exhibit a less pronounced sexual differentiation of several cytochrome P-450-mediated microsomal reactions toward steroid and xenobiotic substrates,[279] suggesting that nodular cells are resistant to the endocrine factor(s) regulating sex-differentiated steroid and xenobiotic metabolism.

As mentioned previously, the hepatic production of IGF-I is controlled by GH, and the finding of a reduced expression of IGF-I in liver nodules and hepatocellular carcinomas[280] prompted a detailed study on the expression of the GH-R in nodules and hepatoma (Table 13).[259] The level of GH-R mRNA expression was found to be reduced to 63 and 4% in nodules and hepatoma, respectively, compared with control liver. The reduction of IGF-I mRNA roughly paralleled the decrease in GH-R mRNA, again indicating the crucial importance of GH-R in the regulation of IGF-I expression. However, when the binding

TABLE 13
Quantitation of GH-R and IGF-I mRNA Levels in Regenerating Liver, Liver Nodules, and Hepatoma

	GH-R mRNA (amol/μg DNA)	IGF-I mRNA (amol/μg DNA)
Control liver	3.6 ± 0.25 (100)	35.7 ± 3.2 (100)
Regenerating liver	2.9 ± 0.28 (80)	17.8 ± 1.9 (50)
Liver nodules	2.3 ± 0.34 (64)	14.3 ± 0.7 (41)
Hepatoma	0.14 ± 0.07 (4)	2.1 ± 0.7 (6)

Data from References 81, 259, and 280.

of radiolabeled GH to membrane GH-R was assessed, the level of ligand binding was even more reduced, and paralleled the mRNA level only after removal of endogenous ligand (Figure 2).[259] Thus, a large fraction of nodular GH-R carry bound GH, resulting in few available receptor sites at the cell surface. These remaining unoccupied receptors were found to be functional with respect to stimulation of IGF-I synthesis following a GH load.[259] It appears likely that the previously described loss of sexual differentiation of liver nodules[279] is related to a decreased number of functional GH receptors at the surface of nodular cells.

The level of GH-R mRNA was much reduced in rat hepatomas (Table 13), and similar low-level expression of GH-R, measured as binding capacity, has also been noted in human hepatocellular carcinomas.[281] The absence of GH-R in liver neoplasms also provides an explanation for the absence of expression of the major urinary protein (MUP), a GH-regulated protein, in liver nodules and hepatomas in the mouse.[282]

To approach the question of whether the increased occupancy of the GH-R is a property specific for nodules, rather than a proliferation-associated event, the expression of GH-R was further analyzed in regenerating liver after partial hepatectomy (Table 13, Figure 2).[275] In this case, GH-R mRNA, IGF-I mRNA, and GH binding was downregulated to a similar extent as in nodules; however, in contrast to nodules, no further increase in GH binding was observed after attempts to dissociate endogenous ligand (Figure 2). This suggests that either very low levels of functional GH-R are produced by the regenerating cells of the liver remnant, or that the endocytosed receptor is rapidly degraded, and for that reason is unable to bind ligand. In any case, harboring a large population of undegraded, ligand-occupied GH receptors suggests that one basic defect in nodular cells resides in the capacity for GH dissociation and/or ligand-receptor degradation.

3. Transforming Growth Factor-β During Hepatic Regeneration and Hepatocarcinogenesis

Transforming growth factor type-β is a dimeric polypeptide of 25 kDa composed of 112 identical amino acid subunits associated by disulfide linkages.[206] TGF-β is synthesized as a larger membrane-anchored precursor form from which the secreted mature form of TGF-β is generated by proteolytic cleavages.[206] Several distinct high-affinity TGF-β receptors have been described, of which at least the 65 kDa and the 110 kDa "low" molecular weight variants (type I and type II receptors, respectively) are required for TGF-β sensitivity.[283] It has been suggested that G-protein coupling is required for the transduction of the TGF-β signal from the type I receptor.[284]

TGF-β is a potent inhibitor of EGF-stimulated DNA synthesis in primary monolayer cultures of rat hepatocytes,[84,285,286] without inhibiting EGF binding or EGF receptor autophosphorylation.[287] This inhibition is modulated by activation of the α_1-adrenergic receptor, which shifts the dose-response curve to the higher concentrations of TGF-β required for inhibition of DNA synthesis.[288]

In the regenerating liver, the synthesis of TGF-β is induced in the liver remnant with an increased message level detectable by 4 h after surgery and the peak level observed at 24 h,[289,290] a time when the major wave of cell proliferation is under way. Interestingly, the induced TGF-β expression occurs in the nonparenchymal endothelial cells and in the periportal area, suggesting a paracrine mode of action.[289-291] Intravenous administration of TGF-β to rats after partial hepatectomy results in a significantly decreased cell proliferation when given in multiple injections during the prereplicative period, although the inhibitory effect appears to be transient.[292] The inhibitory effects of TGF-β appear to be counteracted in regenerating hepatocytes by developing a slight TGF-β resistance.[293] The mechanism of this resistance appears to involve downregulation of TGF-β receptors.[210]

It can be hypothesized that loss of sensitivity to growth inhibition and increased activation of cell proliferation are important factors, both of which contribute to the development of neoplastic cells. Since TGF-β is a potent growth inhibitor for parenchymal hepatocytes, a number of studies have aimed at analyzing the expression of TGF-β during liver carcinogenesis. In nodular and hepatoma cells formed according to the resistant

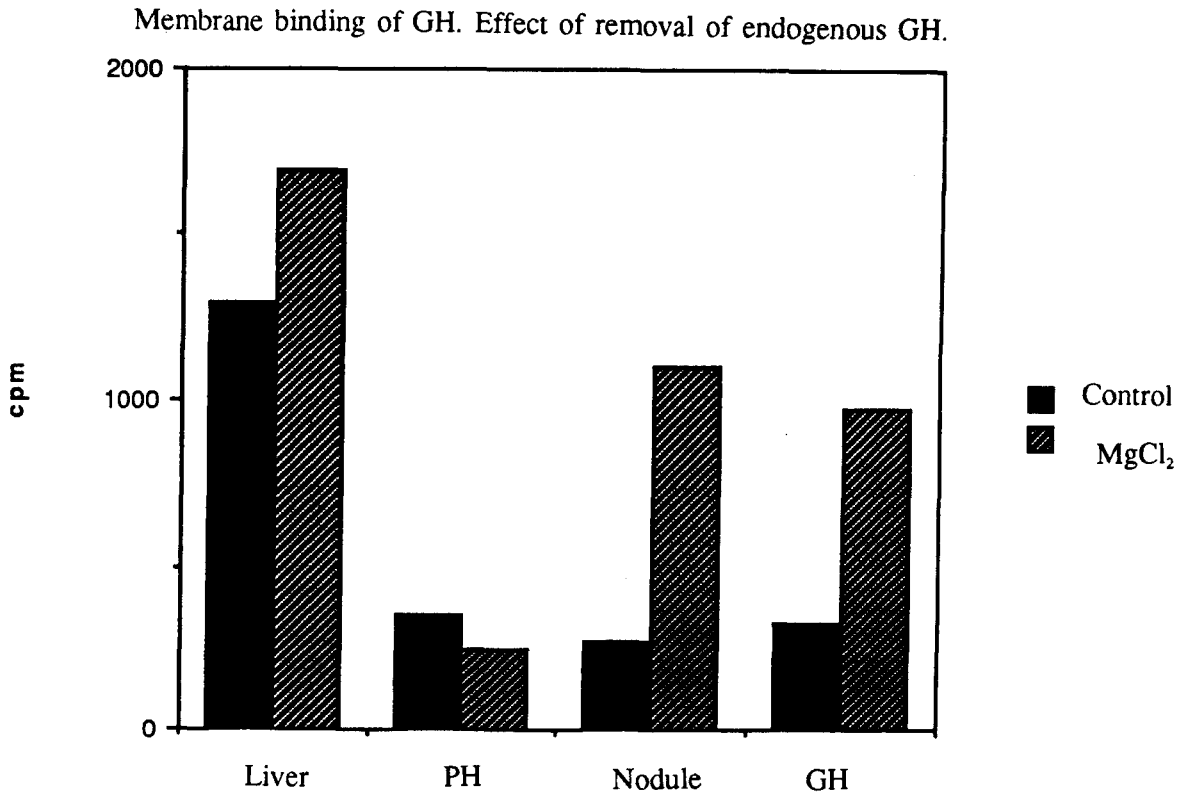

FIGURE 2. GH binding to membrane receptors before and after dissociation of endogenously bound GH. A low-density membrane fraction enriched in Golgi and endosome membranes was isolated from control liver (liver), liver remnants 24 h after partial hepatectomy (PH), liver nodules (nodule), and from liver of rats 16 h after the s.c. injection of 2 mg human growth hormone (GH). Following isolation, membranes were subjected to dissociative treatment by exposure to 4 *M* $MgCl_2$ (hatched bars) or as a control to isotonic sucrose (black bars), and the binding of iodinated human growth hormone was determined. (Data adapted from References 259 and 275.)

hepatocyte model, a similar sensitivity to inhibition of EGF-stimulated DNA synthesis by TGF-β as found in normal adult hepatocytes was noted.[84] In contrast, a rat liver epithelial (RLE) cell line transformed *in vitro* with aflatoxin B_1 was significantly less sensitive to the growth-inhibitory effects of TGF-β compared with the parental untransformed celline.[294] The induction of TGF-β resistance by a short exposure of RLE cells to *N*-nitrosoguanidine was found not to be associated with significant differences in cellular binding of labeled TGF-β.[294] Also in an inmortalized, nontumorigenic oval cell, the sensitivity to growth inhibition by TGF-β was lost upon either spontaneous or *H-ras*-induced transformation.[295] Also in this case, no difference in the TGF-β binding upon transformation was observed, implying an altered postreceptor mechanism in the reduced sensitivity to TGF-β.[295]

Using *in situ* hybridization, TGF-β_1 transcripts can be detected in periductular and endothelial cells of the portal region in normal liver, in perinodular oval cells during the early stages of liver carcinogenesis, and in mesenchymal cells surrounding fulminant hepatocellular carcinomas.[290] TGF-β can induce the differentiation of rat liver epithelial cells, similar to oval cells along the hepatocyte lineage, suggesting a physiological role for TGF-β in liver stem-cell differentiation.[296]

In conclusion, proliferative hepatocytes during compensatory liver growth appears to escape part of the inhibitory influence of TGF-β on cell proliferation by downregulation of TGF-β receptors and additional mechanisms involving adrenergic receptors. During carcinogenesis, nodular and hepatoma cells *in situ* exhibit an unaltered sensitivity to TGF-β-mediated inhibition of cell

proliferation. The absence of TGF-β expression in the nodular or carcinoma cells in combination with high expression in the cells surrounding nodules/tumors may generate a situation with differential mitoinhibition and a relatively higher rate of nodular/tumor cell proliferation compared with the surrounding TGF-β-inhibited cell compartment. This relative growth advantage could be an important factor underlying the expansion of the tumor compartment.

4. Heparin-Binding Growth Factors During Hepatic Regeneration and Hepatocarcinogenesis

The best known heparin-binding growth factors, besides TGF-β, that have been implicated in the growth regulation of liver cells are the acidic and basic fibroblast growth factors and the hepatocyte growth factor.

a. Fibroblast Growth Factors

The fibroblast growth factor (FGF) family consists of seven related gene products that stimulate and inhibit the proliferation of cultured rat hepatocytes and differentiated human hepatoma cells.[297] The most well-known members are acidic FGF (HBGF-1/endothelial cell growth factor), an acidic protein of 17.4 kDa, and basic FGF (HBGF-2), a basic protein of 18 kDa. The members of the FGF family interact with a family of FGF receptors, which are formed by combinations of three amino-terminal domain motifs, two juxtamembrane domain motifs, and two intracellular carboxy-terminal domain motifs.[297] Given the complexity of this growth factor-receptor system, it is not surprising the biological responses following ligand stimulation of the receptors also appear to be complex. Stimulation of human hepatoma HepG2 cell proliferation by aFGF was found to be correlated with high-affinity binding to a 130 kDa receptor, whereas inhibition of proliferation at higher concentrations of aFGF was associated with the low-affinity binding to a receptor species of similar size.[298] In regenerating liver, the appearance of a high-affinity aFGF receptor, in addition to the low-affinity binder also present on quiescent liver cells, was observed.[298] Increased expression of aFGF mRNA was noted already at 4 h posthepatectomy in the liver remnant and was sustained for 7 d.[299] Since EGF inhibits the proliferative response to aFGF in isolated hepatocytes concurrent with the appearance of low-affinity aFGF binding sites,[299] it was hypothesized that auto- or paracrine aFGF expression initiates a proliferative response in the remnant cells and that this stimulating effect is abrogated when the remnant cells initiate the expression of TGF-α.[224] As previously noted, increased TGF-α expression occurs concomitantly with a downregulation of EGF-receptor expression.[210] Interestingly, aFGF has been shown to downregulate EGF binding sites by a rapid PKC-independent pathway in mouse Swiss 3T3 cells.[300]

b. Hepatocyte Growth Factor

Hepatocyte growth factor (HGF or hepatopoetin A) was initially isolated from the serum of partially hepatectomized rats and was shown to stimulate DNA synthesis in serum-free primary cultures of quiescent hepatocytes.[301,302] The purified human, rabbit, and rat HGF are acidic, heparin-binding, heterodimeric proteins of ≈80 kDa, consisting of 70 kDa (α) and 35 kDa (β) subunits.[303,304] The mitogenic effect of rabbit HGF was found to be completely inhibited by TGF-β and was additive to the stimulatory effect of EGF + insulin on quiescent rat hepatocytes.[304] The primary structure of rat[305] and human[306] HGF deduced from partial cDNA clones showed that a single open reading frame encoded both HGF subunits in a 728 amino acid long protein. Thus, the heterodimeric structure of the mature HGF is generated by proteolytic cleavage, presumably by a trypsin-like protease. Curiously, there is an overall 38% homology between HGF and plasminogen, and the HGF α-subunit has "kringle" structures similar to those found in plasminogen. Additionally, the β-subunit shows a significant homology with a number of serine proteases, including plasmin.[305,306] However, replacement of active-site residues suggests that the HGF-β subunit is catalytically inactive. The HGF mRNA is highly expressed in rat lung and liver, but appears to be present in most tissues.[305] HGF mRNA levels are upregulated in livers of hepatotoxin-treated rats,[307] where the message is localized in

nonparenchymal endothelial and Kupffer cells.[308] Since no increase in HGF mRNA was observed in regenerating livers after partial hepatectomy,[308] it appears likely that HGF is involved in a tissue repair mechanism different from regenerative liver growth.* Recently, purification and cDNA cloning revealed the identity of a lung fibroblast-derived mitogen as a variant of HGF.[309] This HGF-like mitogen was suggested to play a role as a paracrine mediator of the proliferation of melanocytes and endothelial cells.[309] This protein, as well as HGF, interacts with and stimulates tyrosine phosphorylation of a 145 kDa receptor on the surface of target cells.[309] The HGF receptor has recently been identified as the β-subunit of the *c-met* protooncogene product.[310]

B. Regulation of Cell-Surface Receptor Expression in Proliferating Cells

Theoretically, a large number of regulatory mechanisms can be responsible for regulating the cell-surface expression of functional membrane receptor proteins. These include regulation of receptor gene transcription; transcript stability and receptor protein synthesis; intracellular transport with associated posttranslational processing, such as disulfide formation, glycosylation, etc.; and, as indicated in Figure 2, receptor endocytosis, downregulation, and various postreceptor mechanisms, such as phosphorylation, proteolysis, etc., as well as sorting of internalized receptors for recycling or degradation, including ligand dissociation, receptor inactivation, and/or degradation. In the cell, all these regulatory mechanisms, if operative, are most likely inter regulated to maintain an appropriate expression of functional cell-surface receptors that is adequate for the normal functioning of the cell at any given time. We will concentrate our discussion on regulatory mechanisms involving receptor synthesis and receptor and/or ligand degradation, mainly due to the lack of information regarding other regulatory mechanisms in cells undergoing carcinogenesis.

Cell proliferation is stimulated or suppressed by activation of one or several growth factor receptors, usually as a result of growth factor binding to its receptor. The activated receptor transduces signal(s) intracellularly that eventually lead to a proliferative response. In a regulated growth situation, it is required that the critical signal(s) are attenuated once a sufficient number of new cells have been generated. However, in unscheduled growth, such as in tumor development, it is reasonable to assume that the balance between regulatory principles that, on the one hand, generate intracellular signals and, on the other hand, terminate these signals are permanently altered, due to mutational events, in the direction of an increased proliferative potential.

Regarding the growth-regulatory cell-surface receptors, i.e., EGF- and GH-receptors, that has been described in previous sections of this review, one consistent feature of the regulation occurring during carcinogenesis relates to a decreased cell-surface receptor expression, due primarily to diminished levels of receptor mRNA leading to decreased receptor synthesis. It can be speculated that this represents an attempt to dampen the proliferative pressure induced by, for instance, autocrine and/or paracrine growth-stimulatory factors.

A number of observations also suggest that postreceptor modulation contributes to the downregulated receptor responsiveness in the preneoplastic cell. The idea that nodular cells exhibit an altered capacity for proper acidification of endocytic, and possibly exocytic, vacuolar cytoplasmic membrane compartments was initially surmised from the examination of the nodular expression of transferrin and asialoglycoprotein receptors (Table 14).[15,235] Interestingly, nodular cells are iron deficient (see below) and express high transferrin receptor levels, similar to cells treated with iron chelators,[311] or weak bases leading to elevation in intracytoplasmic pH[312] or in mutant cells deficient in the capacity for vacuolar acidification.[313] The scenario underlying this iron-deficient phenotype of nodular cells is described in detail in a separate section.

Two lines of evidence indicate that the vacuolar pH of nodular cells is elevated; first, the

* After the submission of this review, two articles were published showing increased hepatocyte growth factor (HGF)mRNA levels in regenerating liver peaking at 12 h[438] or 24 h after partial hepatectomy.[439]

TABLE 14
Number of Transferrin (Tf)- and Asialoglycoprotein (ASOR) Binding Sites and Dissociation Constants in a Golgi/Endosome Fraction from Regenerating Liver and Liver Nodules

	Tf		ASOR	
	N_0	K_D	N_0	K_D
Control liver	0.61 ± 0.07 (1)	0.53	18 ± 3 (1)	0.38
Regenerating liver	1.4 ± 0.1 (2.3)	0.51	12 ± 1 (0.67)	0.39
Liver nodules	4.9 ± 0.5 (8.0)	0.41	4.4 ± 0.6 (0.24)	0.34

Note: For explanations see Table 12.

Data from References 15 and 237.

activity of the vacuolar-type H^+-ATPase was significantly lower in isolated endocytic vesicles from nodules compared to liver and Figure 4;[16] second, following intraperitoneal administration, the accumulation of the weak base chloroquine in nodular endosomes and lysosomes was clearly lower than in the corresponding fractions from normal liver (Figure 4), indirectly demonstrating that the luminal environment of these cytoplasmic compartments is more alkaline in nodules compared with liver.

Interestingly, lysosomes and autophagic vacuoles from nodules exhibit both a deficiency of certain hydrolytic enzymes and a reduced capacity for protein degradation.[314] There is reason to believe that a defective acidification mechanism may play a causative role in decreased nodular endosomal/lysosomal catabolism by, for example, missorting of lysosomal hydrolases transported by mannose-6-phosphate (M-6-P) receptors to the secretory pathway.[315,316]

As it has been shown that DNA synthesis and cell proliferation induced by EGF is inhibited as the pH of acidic, vacuolar membrane compartments is elevated,[317] it is tempting to speculate that the decreased vacuolar acidification of preneoplastic (and possibly also neoplastic) cells represents yet another mode of downregulating the responsiveness to growth regulation, in addition to decreased receptor synthesis, as discussed previously. Whether the altered vacuolar acidification leads to changes in receptor signaling, recycling, or degradation is not yet known, but answering such questions will certainly provide important information regarding the role of various postreceptor mechanisms in the regulation of cell proliferation. Since activation of *ras* oncogenes following fibroblast transformation appears to regulate the acidity of vacuolar compartments toward a more alkaline pH,[318] a potential role of oncogene activation for the regulation of cell-surface receptor expression is clearly indicated.

C. Proteins of Drug Metabolism and Drug Resistance

Liver nodules deal with xenobiotic compounds in a way that favors the excretion of detoxified metabolites in a more efficient way than normal liver cells.[319-321] Phase I monooxygenase reactions are very slow due to downregulated cytochrome P-450 levels. The slow stream of metabolites from phase I can readily be conjugated by the elevated phase II enzymes before reactive species have reacted with cellular DNA and RNA. The pattern of alteration of drug-metabolizing enzymes in liver nodules has been reviewed earlier[58] and will not be covered in detail in this presentation. Tables 15 and 16 summarize the amounts and activities of drug-metabolizing enzymes in liver nodules. It is obvious that drug handling is not entirely a membrane-related phenomenon and, therefore, is not relevant to this review. However, we believe that drug metabolism and drug resistance are essential for the neoplastic growth and development, justifying a brief overview of drug-metabolizing enzyme systems.

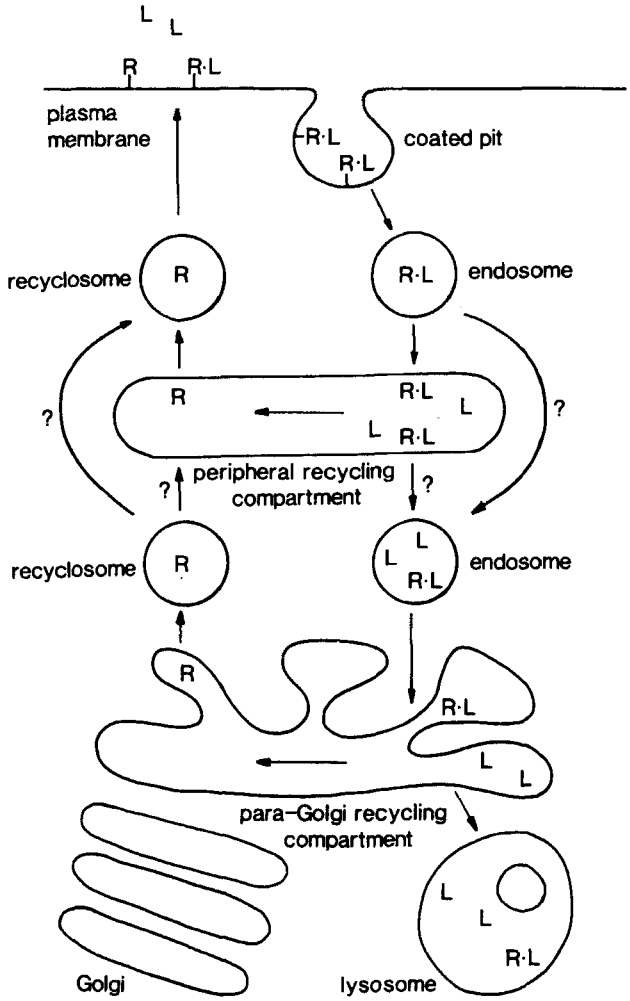

FIGURE 3. Schematic representation of intracellular membrane compartments involved in receptor-mediated endocytosis. R = receptor, L = ligand, RL = receptor-ligand complex.

1. Phase I Metabolism

In Table 15 the relative amount of cytochrome P-450 and the activities of different mixed-function oxidases are summarized. With the possible exception of some phenobarbital-inducible forms (CYPIIB1/2),[322,323] which are not altered, all other cytochrome P-450 isoenzymes thus far investigated are decreased (see Reference 58 for references). The variability in the relative expression indicates some specificity in the regulation of the cytochrome P-450 gene family and makes more general mechanisms of depression less probable, i.e., iron deficiency, low heme synthesis, and low levels of heme (see below). Immunostaining techniques, with antibodies to the enzyme protein (apoenzyme), confirm that the low nodular activity is due to a decreased content of the specific cytochrome.[322] Phenobarbital[322] and 3-methylcholantrene[324] treatment of nodule-bearing rats induce the nodular P-450 content, indicating that the nodules are capable of responding to xenobiotic exposure to a similar extent as normal liver. Interestingly, Chen and Eaton[325] recently found that phenobarbital induced CYPIIB1/2 differently in liver nodules compared with surrounding tissue in the resistant hepatocyte model, leading to a further increase in the difference in the expression of this cytochrome between nodules and surrounding tissue. In their study, the nodular content of CYPIIB1/2 did not increase, nodular pentoxyresorufin *O*-

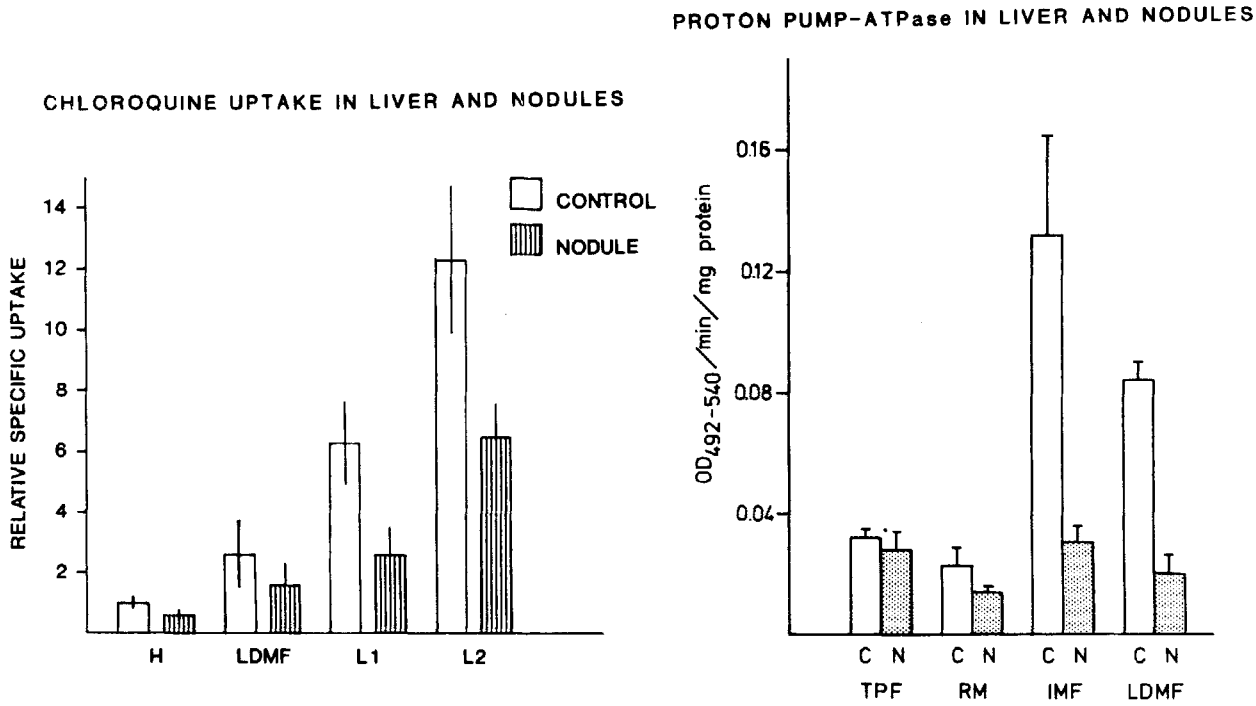

FIGURE 4. Uptake and retention of chloroquine and proton pump-ATPase activity in different membrane fractions of liver and nodules. Data from Reference 16. H = homogenate, LDMF = low-density Golgi/early endosome fraction; L1 = prelysosomal late endosomes; L2 = lysosomes; TPF = total membrane fraction; RM = microsomes; IMF = intermediate density fraction; C = control; N = nodules.

dealkylation increased only 2.4-fold, while the surrounding enzyme activity increased 31-fold. In contrast, in nodules produced with aflatoxin B_1, phenobarbital induced CYPIIB1/2 and the dealkylation reaction considerably (30-fold) and equally in nodules and surrounding tissue. In the latter study, no effect of phenobarbital was seen on the growth of the lesions, indicating the importance of low phase I activity and maintained nodular/surrounding differential expression for nodular growth. The microsomal activity of N-OH-2-acetylaminofluorene hydroxylase in liver nodules is very low, with 4% of normal activity remaining, clearly showing that drug-activating pathways of drug metabolism are also decreased in liver nodules.[326]

2. Phase II Metabolism

Table 16 summarizes results from studies on cytosolic and membrane-bound enzymes that participate in the metabolism of xeno- and endobiotic substances, other than cytochrome P-450, but are usually active on products from phase I reactions catalyzed by monooxygenases. In general, these enzymes are deactivating by increasing water solubility and excretion of the compound, and they are referred to as phase II enzymes. The relative activities of these enzymes are elevated in the nodules (Table 16; see References 27 and 58 for references), with the exception of sulfotransferase.[327] For several phase II enzymes, the appearance of tumor-specific isoenzymes has been suggested, but careful isolation and characterization, including cloning and sequencing of cDNAs, have not confirmed this hypothesis. However, Bock et al.[328] found that nodules contain a 55-kDa, 3-methylcholantrene-inducible UDP-glucuronyltransferase and, in addition, a novel 53-kDa variant. On the bases of immunological and functional properties, the 53-kDa species most likely represents a posttranslationally modified variant of the inducible form, probably altered in the high-mannose oligosaccharides.[328] Also in other cases, nodule- and tu-

TABLE 15
Relative Levels and Activities of Phase I Drug-Metabolizing Enzymes in Microsomal Preparations from Rat Liver Nodules

	Relative value nodular/normal
Cytochrome P-450 (diff. spect)	0.20
Cytochrome P-450 IIB1/2 (immunoblot)	1
Aminopyrene *N*-demethylase	0.24
Benzo(a)pyrene monooxygenase	0.05
Ethoxyresorufin *O*-deethylase	0.12
Ethoxycoumarin *O*-deethylase	0.45
Androstendione 16α-hydroxylase	0.22
Androstendione 6β-hydroxylase	0.21
2-Acetylaminofluorene *N*-hydroxylase	0.04

Note: Normal liver = 1.

See Reference 58.

mor-specific proteins have proven to be normal, inducible enzymes, i.e., preneoplastic antigen-epoxide hydrolase;[329] tumor-type aldehyde dehydrogenase-aldehyde dehydrogenase class 3, inducible with methylcholantrene;[330] and 21-kDa cytosolic polypeptide[331]-glutathione S-transferase 7-7 (GST-π).[332,333] The later enzyme is, however, not expressed in normal rat liver, but is highly and consistently expressed in rat liver nodules. GST-π is now regarded and used as a marker for liver nodules,[334] and its appearance in drug-resistant human tumors has enabled its use as a potential marker for drug resistance.[69,335]

In the bioactivation of 2-acetylaminofluorene, sulfation of the *N*-hydroxylated compound is the most important metabolic route, forming the labile sulfate ester that is a precursor for the ultimate reactive molecule, the nitrenium ion.[336-338] It has also been shown that this metabolic activation pathway is responsible for the hepatotoxic effect of the compound in rodent liver.[339-341] Interestingly, the activity of *N*-hydroxy-2-acetylaminofluorene sulfotransferase in the cytosol of liver nodules is much reduced[58,327,342] and constitutes the only example of a conjugating, phase II enzyme that is not overexpressed in liver nodules. Kroese et al. showed that inhibition of sulfotransferase with pentachlorophenol (PCP) reduced the binding of *N*-hydroxy-2-acetylaminofluorene in the cells surrounding liver foci[343] and that PCP eliminated the difference in the covalent binding of the substance between initiated and noninitiated cells. In a subsequent paper,[344] it was shown that focal growth during promotion in the resistant hepatocyte model was reduced by PCP treatment, leading to the conclusion that *N*-sulfation is responsible for the *N*-hydroxy-2-acetylaminofluorene-mediated outgrowth of diethylnitrosamine-initiated hepatocytes to preneoplastic foci. The low sulfation capacity of female and growth hormone-feminized rats has also been suggested to explain their relative insensitivity to nodule and tumor formation using the resistant hepatocyte model.[278] Recent experiments show that female rat liver and feminized male rat liver are in fact less sensitive to the mitoinhibitory effect of 2-acetylaminofluorene and thus are capable of responding to regenerating growth promotion, reducing the selective growth pressure on the focal lesions.[76]

Enzyme alterations described in liver nodules are constitutive and are not dependent on the continuous presence of drug to maintain the induced expression. Studies on different phase II enzymes (glutathione S-transferase, UDP-glucoronyltransferase, DT-diaphorase, aldehyde dehydrogenase) show that the reversible induction, proportional to the dose of inducer and dependent on its presence, can be separated from the persistent alteration of enzyme expression in the nodules.[324,328,345,346] Exposing nodule-bearing rats to inducers such as 3-methylcholantrene result in superinduction of glutathione S-transferases, DT-diaphorase,[345] and aldehyde dehydrogenase[324,346] in liver nodules. After termination of the treatment, enzyme expression decreases to the constitutive high levels found in the nodules.[324] Blot analysis of mRNA levels suggests pretranslational enhancement to be responsible for both the reversible and constitutive induction. Work by Williams et al.[347] and Ding et al.[348] strongly suggests that the high nodular expression of NAD(P)H:quinone reductase and epoxide hydrolase can be explained by gene hypomethylation. DNA undermethylation as a mechanism for the permanent elevation of gene expression is supported by recent studies showing that the c-*myc* protooncogene[349] and the HMG-CoA reductase gene[194] are both hypomethylated in liver nodules. Furthermore, DNA undermethylation has been

TABLE 16
Relative Activities of Phase II Drug-Metabolizing Enzymes in Preparations from Rat Liver Nodules

			Relative value nodular/normal
UDP-glucuronosyl transferase	(UDP-GTI)	(Microsomes)	5.8
	(UDP-GTII)	(Microsomes)	1.0
Glutathione S-transferase	1-1	(Cytosol)	3.2
	2-2	(Cytosol)	3.0
	3-3	(Cytosol)	3.1
	4-4	(Cytosol)	0.65
	7-7	(Cytosol)	1.2% of cyt. protein/ not detect. in normal
		(Cytosol)	
		(Microsomal)	1.0
N-OH-2-AAF sulfotransferase		(Cytosol)	0.063
Epoxide hydrolase		(Microsomes)	5.2
		(Cytosol)	1.0
DT-diaphorase		(Cytosol)	13
Aldehyde dehydrogenase	(benzald:NADP)	(Cytosol)	40–60
	(propionald:NAD)	(Cytosol)	2
Gamma-glutamyl transferase		(Membrane)	20–40
MDR-glycoprotein P-170 (*mdr*-encoded cDNA sequence)			3–12

Note: Normal livaer = 1.

See Reference 58. Gamma-glutamyl transferase activity is from Reference 8; *mdr*-expression is from Reference 387.

described in tumors and tumor cell lines.[350,351] Phenobarbital treatment of normal rat liver, on the other hand, reversibly induces epoxide hydrolase without evidence of gene hypomethylation.[352] No change in the methylation status of CYPIA1 and CYPIIB1 genes were noted in rat liver after treatment with lead nitrate, which reversibly decreases the expression of these cytochromes.[353]

3. Gamma-Glutamyl Transferase

Gamma-glutamyl transferase is a membrane-bound glycoprotein that catalyzes the hydrolysis of gamma-glutamic acid from peptides and transfer to other amino acid and peptide acceptors.[354,355] The enzyme is, for example, involved in the breakdown of glutathione (L-gamma-glutamyl-L-cysteinyl-glycine), a cytosolic constituent of all living cells. The enzyme is also involved in transmembrane amino acid and peptide transport and detoxification in the liver,[354,356] in ammoniagenesis in the kidney,[357] and in the production of leukotrienes.[358] In the liver,[359] skin,[360] and colon[361] gamma-glutamyl transferase shows an oncofetal pattern of expression with transient high activities in fetal tissue and low but inducible expression in adult tissue. In the adult liver, bile duct cells and occasional periportal hepatocytes express gamma-glutamyl transferase activity, particularly in the bile canalicular area.[362] Various drugs, for example, phenobarbital, reversibly induce periportal gamma-glutamyl transferase activity in the liver. Marked and constitutive elevation of enzyme expression has been described in chemically induced carcinomas and their early prestages from many different tissues in rodents.[363-368] Also in human epithelial neoplasia, a tumor-associated pattern of expression

has been reported.[40,369-372] In experimental carcinogenesis, gamma-glutamyl transferase expression is regarded as a marker for preneoplastic and neoplastic lesions. Techniques are currently being developed for also using this enzyme as a marker in human medicine in the diagnosis of preneoplasia and to estimate the risks of malignant transformation.

Comparative biochemical studies of liver and liver nodules in the rat confirm a 20- to 40-fold elevated expression of membrane-bound gamma-glutamyl transferase activity in liver nodules.[8] The highest activities were measured in isolated plasma membranes, but the enzyme was also found in endoplasmic reticulum membranes, as well as in cytosol and serum from nodule-bearing rats. The relative subcellular distribution pattern did not differ between normal liver and liver nodules.

Interestingly, gamma-glutamyl transferase isolated from rat hepatoma cells is glycosylated differently than the normal counterpart,[373] but the functional consequences of this molecular alteration are not known. Comparative studies on the *N*-linked oligosaccharides in gamma-glutamyltransferase of rat liver and hepatoma show structural alterations consisting of the addition of a "bisecting" *N*-acetylglucosamine in a β1→4 linkage to the β-linked mannose of the trimannosyl core.[373] The specific glucosaminyltransferase responsible for this sugar addition, UDP-glcNAc:glycopeptide β4-*N*-acetylglucosaminyltransferase III,[374] is not expressed in normal adult liver, but is present with substantial activity in liver nodules generated by different models of liver carcinogenesis[375-377] and hepatocellular carcinoma.[375] The addition of a "bisecting" *N*-acetylglucosamine residue appears to prevent further modification of the α6-branch of the trimannosyl core, thus explaining why hybrid-type oligosaccharides are often associated with a bisecting residue.[378] In light of the previously described reduction of dolichyl phosphate levels and cotranslational addition of *N*-linked oligosaccharides,[159] it is noteworthy that the *N*-acetylglucosaminyltransferases I and II, responsible for the addition of *N*-acetylglucosamine to the α3 and α6 mannose residues of the trimannosyl core, respectively, are depressed in liver nodules, whereas the activity of the branching enzymes *N*-acetylglucosaminyltransferases IV and V are elevated.[375] These alterations of *N*-linked oligosaccharide biosynthesis in liver nodules would most likely result in fewer *N*-linked oligosaccharides with an increased branching pattern on secreted and cellular glycoproteins.

The enrichment of gamma-glutamyl transferase in the cell membrane, particularly the bile canalicular area of nodular and hepatoma cells and its function as a transmembrane transporter, may be of relevance for the resistant behavior of the cells. Glutathione, a substrate for the transferase, is fivefold elevated in liver nodules over normal levels,[2,173] and the constitutive induction of gamma-glutamyl transferase in nodules may thus at least partly be related to elevated substrate levels. In a recent series of articles, Stenius et al.[379-381] showed that, in an *in vitro* model, cells overexpressing gamma-glutamyl transferase are more resistant to glutathione depletion and oxidative stress than normal hepatocytes. This phenomenon was observed only when the cells had excess extracellular glutathione, indicating that the involvement of the membrane-integrated enzyme is involved in extracellular glutathione breakdown. The released and internalized cysteine is necessary and rate limiting for the intracellular synthesis of the nucleophilic tripeptide.[382] As for other constituents of the resistant phenotype, known functional alterations related to the specificity of toxic exposure or the resistance profile cannot be defined.

4. The Multidrug Resistance Glycoprotein

The multidrug resistance P-glycoprotein is a 170-kDa transmembrane glycoprotein, functioning as an efficient, pump transporting a multitude of lipophilic chemotherapeutic drugs and carcinogens out of the cell.[67,383] Recent work by Fairchild et al.[384] and Thorgeirsson et al.[385] have shown elevated levels of *mdr* mRNA and P-glycoprotein in liver nodules, and it is reasonable to assume that this protein also participates in maintaining the functional drug resistance of liver nodules. However, Huitfeldt et al.[386] pointed out that the P-170 glycoprotein expression in the resistant hepatocyte model of chemical carcinogenesis was not different in the nodules compared to the surrounding tissue, indicating that overexpression

of the *mdr* protein was neither a necessary nor a sufficient prerequisite for cell proliferation during 2-acetylaminofluorene mitoinhibition. This suggests that other factors related to drug handling must be considered. Eriksson et al.[319] and Spiewak-Rinaudo et al.[320] showed, in studies of the kinetics of 2-acetylaminofluorene uptake and excretion in liver and liver nodules, that equal amounts of the parent compound were unspecifically retained in the lipid phases in nodular and normal liver cells. For lipophilic substances, such as 2-AAF, uptake kinetics[319] suggest that diffusion and partition between hydrophobic sites in serum and in the cell is the most important uptake mechanism. Blood and tissue distribution studies[319] showed that the free pool of unmetabolized substance in the liver was communicating with the parent compound in serum and body fat pools. The intracellular radioactivity was distributed according to the relative lipid content of the membrane compartment, with one notable exception, the endoplasmic reticulum, where, in addition, 2-AAF was retained in proportion to the amount of cytochrome P-450. Furthermore, no parent 2-AAF excretion to the bile was observed, only 2-AAF metabolites. The rate of bile excretion was also proportional to the rate of phase I metabolism, being slow in nodules, more rapid in normal rats, and very rapid in 3-methylcholantrene-treated rats. From these data it appears unlikely that extrusion of unmetabolized 2-AAF via a membrane pump can explain differences between nodular and surrounding cells in the amount of covalent binding of the drug to cellular macromolecules. Rather, since only reactive metabolites of 2-acetylaminofluorene will bind to cellular constituents, or be excreted, phase I and phase II metabolism seem to be the major rate-limiting steps in determining the cellular toxicity of 2-AAF.

Pharmacokinetic data from studies of 2-acetylaminofluorene in the resistant hepatocyte model and the known pattern of drug-metabolizing enzymes can be used to construct a probable model for biotransformation. In spite of the fact that the intracellular concentration of parent compound was identical in nodular and surrounding tissues, the formation of hydroxylated metabolites was decreased in nodules,[387] indicative of a reduced phase I metabolism. Drug excretion via the bile was slower in nodule-bearing rats than in normal rats, and instead increased amounts of conjugated metabolites were found in the urine.[320] The net effect on metabolite flow was a relative increase of the excreated fraction of metabolites formed and a decrease in the fraction of metabolites covalently bound to intracellular macromolecules.[321] A slow phase I and a high-capacity phase II metabolism can explain the diminished number of 2-acetylaminofluorene DNA adducts in liver foci and nodules compared to the surrounding liver cells[388] in the resistant hepatocyte model.

The phenotypic alterations in drug handling have been studied extensively in many different experimental models where the nodules were produced by different protocols, using different initiators and different promotion regimens.[1,2] In all rat models thus far investigated, a similar pattern of alterations as described above have been found, even in the absence of chemical carcinogens, i.e., in the orotic acid model or in the model of dietary choline-methionine deficiency. It is obvious that not all of the different alterations participate in the protection of the cells in each particular case. The stereotypic reaction, with a multitude of metabolic alterations involved, is instead consistent with a programmed escape process switched on by a threatening, noxious environment.[1] In malignant tumors, this complex phenotypic switch could be responsible for a broad and unspecific multidrug resistance.

D. Membrane Redox and Cellular Iron Transport

1. The Iron-Deficient Phenotype

It was recognized early that liver nodules did not accumulate iron in a situation of iron excess. When the surrounding liver cells stored iron in intracellular ferritin granules, nodular cells came out as iron-negative islands on histochemical sections stained for iron.[389] It was subsequently observed that many iron-containing and iron-dependent enzymes and processes were depressed in the nodules compared with normal or surrounding tissue. Reduced levels of heme-containing enzymes, such as cytochrome P-450, cytochrome b_5, tryptophane 2,3-dioxygenase, and catalase, as well as cellular heme, heme-binding protein, and total cellular iron, have been dem-

onstrated in liver nodules (see Reference 27 for references). Roomi et al.[3] and Stout and Becker[390] noticed an increased activity of heme oxygenase, a heme-degrading activity, and a decrease in heme-synthesizing activity, expressed as the activity of 5-aminolevulinic acid synthetase (ALA-synthetase). In spite of reduced ALA-synthetase and reduced iron uptake, heme synthesis in hepatic carcinomas appears to be normal.[391]

In our attempt to define molecular mechanisms involved in the reduction of nodular iron content, transferrin receptor expression was characterized in nodular cells.[15] Binding studies showed an increased number of diferric transferrin binding sites with a normal affinity constant. Studies of iron uptake in nodule-bearing rats *in vivo* showed a decreased nodular uptake of iron after intraportal injection of ^{59}Fe-diferric transferrin.[15] Endocytosis of diferric transferrin *in vitro* using isolated nodular cells showed an initial high rate of endocytosis, followed shortly by decreased uptake, leaving a higher proportion of ligand remaining on the cell surface compared with normal hepatocytes.[392] Exocytosis and recycling of free receptors were slower than in normal controls, resulting in a slow restoration of cell-surface ligand binding capacity. An abnormal processing of endocytosed ligand, due to deficient endosomal acidification resulting in insufficient dissociation of transferrin-bound iron,[16] probably contributes to the slow iron uptake and low intracellular iron content in preneoplastic liver cells.

Receptor-mediated endocytosis is not the only mechanism by which cells take up iron (Figure 5).[393] Iron also undergoes frequent redox cycles while moving from the extracellular to intracellular compartments, as well as between different intracellular locations. Based on experimental evidence from studies of cellular iron uptake *in vitro,* the initial event in cellular iron handling is binding of diferric transferrin to a receptor on the cell surface. From this point, there are at least two ways to incorporate iron into the cell. (1) In the redox model the ferric iron in transferrin is destabilized at the cell surface by conformational alterations in receptor-bound transferrin,[394] possibly in combination with local acidification in the microsphere of the receptor by the Na^+/H^+-antiport. The iron is reduced to ferrous iron due to a transplasma membrane NADH-dependent diferric transferrin reductase.[395] The ferrous iron is transported across the membrane, presumably carried by a ferro-specific membrane binder,[396] to the cytosolic side, where cytosolic iron acceptors such as ferritin bind the iron, which is reoxidized to ferric form. Release from ferritin requires reducing conditions.[397] (2) In the receptor-mediated endocytosis model, diferric transferrin-transferrin receptor complexes are internalized by the process of endocytosis. In the endocytic compartments, the affinity of iron to the binding protein is lowered due to conformational changes in receptor-bound transferrin[394] and acidification of the vesicular compartment, and the iron is reduced by an endosomal reductase.[397] After reduction, the ferrous iron is transported across the endosomal membrane to reach iron binders in the cytosol.

Reduced iron is rapidly oxidized by oxygen. In liver cells, oxygen affects reductive iron uptake, but not the receptor-mediated endocytic uptake of diferric transferrin.[399] Based on the differential oxygen inhibition of iron uptake, it has been postulated that below 1 μM of diferric transferrin, endocytic iron uptake predominates. Above this concentration, the reductive process on the plasma membrane plays an increasing role, reaching a maximum level at 40 μM diferric transferrin.[400-403] Since the serum concentration of diferric transferrin is higher than required for saturation of the high-affinity receptor (dissociation constant 0.1 to 0.5 μM), it is reasonable to assume that the plasma membrane reductive process participates in liver-cell iron uptake.[402]

Both models for iron uptake are dependent on the binding of diferric transferrin to the transferrin receptor, and the regulation of iron uptake can subsequently be modulated by altering the receptor expression. In studies on the regulation of iron uptake, it has been demonstrated that iron-depleted (by desferrioxamine) cells increase the expression of transferrin receptors and reduce the synthesis of intracellular ferritin.[311,404] This regulation was, to a considerable extent, mediated by a cytosolic mRNA binding protein, the iron regulatory factor (IRF), the expression of which is inversely correlated with intracellular iron stores.[405] IRF is an evolutionarily well-conserved protein, found in insects, frogs, fish, birds, and mammals, including humans.[406] IRF binds to the 3′-end of transferrin receptor mRNA, protecting

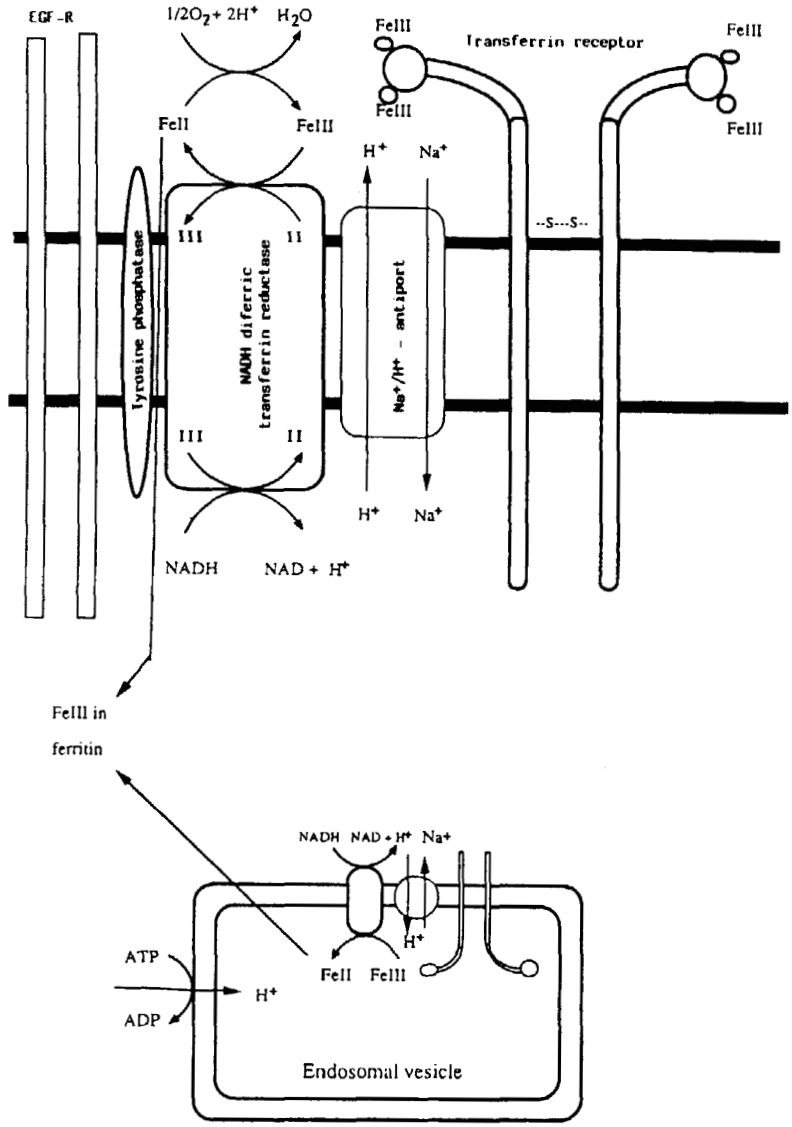

FIGURE 5. Schematic presentation of the NADH transplasma membrane redox system and its postulated relation to the transferrin receptor, iron uptake, Na^+/H^+-antiport, endosomal acidification, tyrosine phosphatase, and receptor phosphorylation. (Data from H. Löw, personal communication and Reference 403.)

the transcript from degradation, leading to stabilization of the messenger, increased concentration, and translation into functional receptors. Furthermore, IRF binds to the 5′-end of ferritin mRNA, which inhibits translation of this transcript. Iron-depleted cells consequently increase their expression of transferrin receptors and reduce their content of ferritin in a complex feedback regulation process. The phenotype of liver nodules, containing low amounts of iron and ferritin, but overexpressing transferrin receptors, is consistent with these findings.

The iron deficiency of liver nodules can be connected to the mechanisms of iron internalization and intracellular transport. In our model, we propose the following sequence of events, based on combined data from studies of receptor-mediated and reductive iron uptake. Liver nodules bind diferric transferrin, which is partly endocytosed. Due to deficient acidification of en-

dosomal vesicles,[16] the destabilization of the iron binding to apotransferrin is not as efficient as in normal cells, making the ferric iron less available for the transmembrane iron reductase. The iron retains its ferric form and remains associated with the receptor. Receptor-ligand uncoupling is decreased and the receptor-bound diferric transferrin recycles to the cell surface. The surface activity of NADH diferric transferrin reductase is decreased in liver nodules, as measured by chelating the ferrous iron formed by bathophenantroline (Nilsson, Eriksson, and Löw, unpublished experiments). Using plasma membrane preparations from the same liver nodules, Morré et al.[407] found a slight reduction of NADH ferricyanide and ferric ammonium citrate reductase activity. It is conceivable that alterations in vesicular acidification and in redox capacity on the cell surface, and possibly also in the endosomes, can explain the reduced iron uptake noted in liver nodule cells.

Tavassoli and co-workers have presented a model in which hepatocytes interact with endothelial cells for their uptake of iron.[408-413] Their data suggest that diferric transferrin is initially transcytosed and desialylated by the endothelial cells. The diferric asialotransferrin reaches the parenchymal hepatocytes via the space of Dissé and is endocytosed via the asialo-glycoprotein receptor. Interestingly, liver nodules express low numbers of asialoglycoprotein binding sites,[237] suggesting an additional mechanism compatible with reduced iron uptake in nodular cells.

E. Transferrin and the Growth of Liver Nodules

Transferrin is an essential growth factor in many cell systems.[414] Inhibition of transferrin binding to the receptor using antibodies against transferrin-binding epitopes on the transferrin receptor inhibits growth.[415] The regulation of transferrin receptor expression is also affected by the growth status of the cell. The number of receptors on the cell surface is higher on proliferating cells,[416] at least partly depending on receptor relocalization.[417] The intracellular iron requirement for iron-containing enzymes — including cytochromes and ribonucleotide reductase — necessary for cell proliferation, has been regarded as the rationale to explain increased receptor expression and transferrin dependence. The discovery that ferricyanide could replace diferric transferrin in melanoma cell cultures[418] showed that the provision of nutritional iron was not the growth-limiting factor. Crane et al.[419] formulated the transplasma-membrane redox concept and showed that diferric transferrin is also an important electron acceptor for NADH-oxidoreductases. In a recent review by Low et al.,[403] the properties and functions of plasma membrane oxidoreductases have been described. Thorstensen and Romslo[393] also reviewed the role of transferrin in cellular growth in a recent publication.

Cell-surface redox activity is associated with alkalinization of the cellular cytosol via proton extrusion through the Na^+/H^+ antiport[420-422] and restoration of the intracellular NAD/NADH ratio.[423] Cellular control of pH and NAD stores[242] is important and rate limiting for growth. Transplasma membrane redox with electron transport to extracellular electron-accepting substrates with appropriate reduction potential is important for the maintenance of growth, permitting homeostasis in the cell. The most abundant extracellular physiological substrate for plasma membrane redox reactions is diferric transferrin.[424] In fact, more ferric iron is used for redox purposes than for iron uptake and intracellular use.[403] The stimulation of transmembrane electron transfer by the addition of diferric transferrin stimulates the growth of many animal cells.[424] The influence of plasma membrane redox on growth regulation is emphasized by investigations showing that growth inhibitors, such as adriamycin and bleomycin, are also potent inhibitors of the redox system.[425,426]

The stimulating effect of iron on cellular growth is thus dependent on the ability of the cell to position diferric transferrin in close association with the electron transport system. This ability is correlated with the number of diferric transferrin receptor sites on the cell surface. NADH diferric transferrin reductase is only one of several transplasma membrane oxidoreductase activities. Others are NADH oxygen oxidoreductase, NADH semidehydro-ascorbate reductase, and NADPH thioredoxin reductase, all utilizing extracellular electron acceptors. Ferricyanide acts as an impermeable artificial acceptor, perhaps for all these activities.[403]

Transplasma membrane redox activity can be

measured with oxygen as the final electron acceptor in the presence of mitochondrial inhibitors, such as rotenone, antimycin A, or cyanide.[427] Morré et al. recently showed that in plasma membrane preparations from normal liver cells, NADH oxidase activity could be stimulated 200% by the addition of 3 μM diferric transferrin.[407] In plasma membrane preparations from liver nodules, however, the basal NADH oxidase activity was at the same level as the iron-induced activity in normal plasma membrane preparations. The addition of diferric transferrin did not further increase the activity. Thus regulation by diferric transferrin was absent in the nodular membranes, where the redox reaction was constitutively activated. A role of plasma membrane redox in growth regulation is intriguing and is supported by the findings in the liver/liver nodule model. In plants growth-factor-dependent NADH oxidation has been described[425] and is associated with growth.

It was shown recently that proliferation of normal liver cells *in vitro,* on a growth-permitting collagen type I biomatrix, was augmented by diferric transferrin.[428] This response was dose dependent in the range of 0.1 to 30 μM. Equimolar concentrations of ferricyanide, an impermeable ferric compound, could replace diferric transferrin. These data support previous findings from studies of other cell systems on the growth-regulatory effect of available electron acceptors for cell-surface redox reactions.[424] Isolated nodular cells showed a higher than normal spontaneous growth rate with a labeling index of 40 to 60%, compared with the normal 10 to 20%, but showed no growth stimulation by extracellular electron acceptors (Figure 6).[429] The behavior of nodular cells in culture thus resemble the activation of the NADH oxidase.[407430] In the scenario, the nodular cells contain an excess of constitutive diferric transferrin bound to the receptor, in close proximity to the reductase, due to their high surface transferrin receptor expression. This alteration would result in an activated plasma membrane redox activity and redox conditions that will not be rate limiting for cell proliferation.

As mentioned above, the importance of the cell-surface redox reaction for cell proliferation is further supported by the findings that adriamycin, even in nonpermeable complexes, inhibits cell growth and the NADH oxidase in parallel.[425,426] Resistance to adriamycin mitoinhibition by nodular cells is expected and could possibly be explained by a higher redox capacity, making the cells less vulnerable to agents interfering with the electron transport complex in the cell membrane. It has to be pointed out that the mechanisms of adriamycin inhibition of redox functions and cell growth are not known. Several alternative redox-dependent steps in receptor-mediated cell functions, including growth regulation, can be suggested as targets for adriamycin. Deregulation of cell-surface redox reactions could, however, help us explain the maintenance of a high spontaneous growth rate and multidrug resistance characterizing neoplastic development.

It was recently shown by Low et al.[431] that ferricyanide and hydrogen peroxide could stimulate tyrosine phosphorylation of the band 3 erythrocyte membrane protein in intact erythrocytes. Furthermore, hydrogen peroxide was reported to stimulate tyrosine phosphorylation in adipocytes.[432] The mechanisms involved are not known, but recent work on protein tyrosine phosphatases in the cell cycle and signal transduction[433] offers interesting possibilities to explore in the future. Protein tyrosine phosphatases catalyze the dephosphorylation of tyrosine phosphorylated proteins, such as growth factor and hormone receptors. One such enzyme, purified to homogeneity from human placenta, is a 48-kDa, membrane-bound protein that is absolutely specific for tyrosyl residues that displays a high specific activity of 40 μmol/min/mg of protein with a low K_m of 20 nM.[433,434] The phosphatase is thus three orders of magnitude more active than the kinases, which necessitates tight regulation to keep up the levels of tyrosine phosphates. The phosphatase is able to dephosphorylate the insulin receptor, which has been autophosphorylated in the presence of insulin, and to inhibit hormone action.[435] Microinjection of the purified enzyme in *Xenopus* oocytes diminishes tyrosine phosphate levels and causes a delay in meiotic maturation.[436]

It is an attractive idea that the kinases and phosphatases act like reins controlling the level of phosphorylation of growth-regulating receptors. The kinases are activated by ligand interaction, but what factors direct the activity of the phosphatases? Phosphatase activity is inhibited

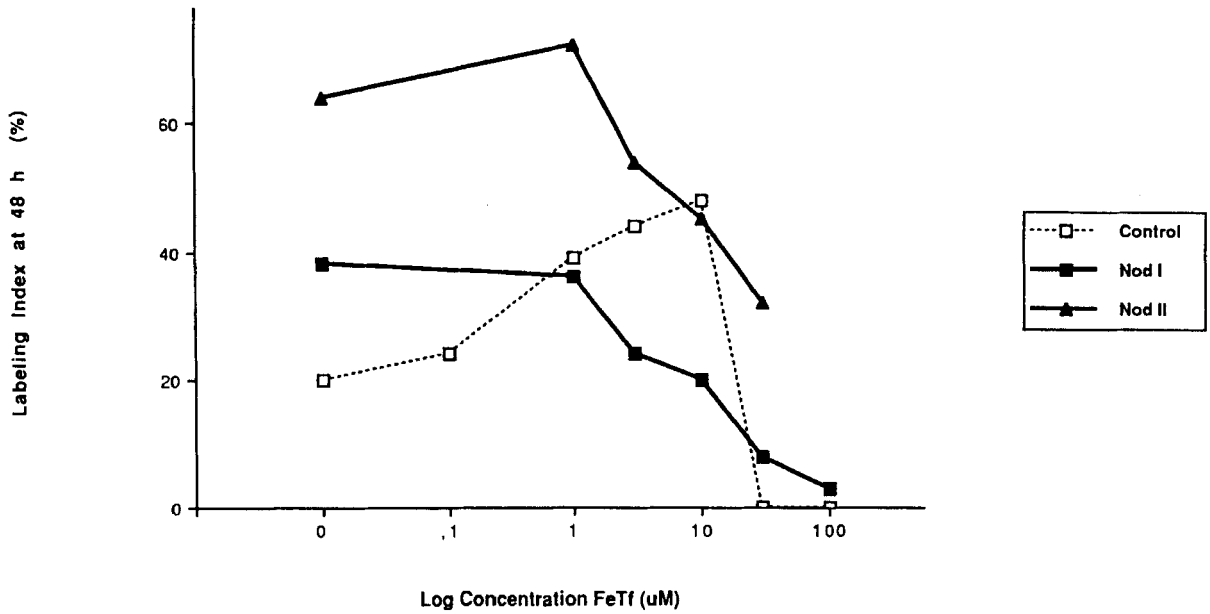

FIGURE 6. The effect of diferric transferrin on the *in vitro* proliferation of hepatocyte from normal liver and liver nodules. (Data from References 428 and 429.)

by nanomolar concentrations of heparin and by vanadate and ferricyanide, which are excellent substrates for the plasma membrane redox system. It is therefore possible that increased redox activity, by inhibiting tyrosine phosphatase activity, contributes to elevate the degree of receptor tyrosine phosphorylation and signal transduction. Overexpression of kinases can lead to cell transformation.[437] Deregulated or hyperactive redox on the cell surface may have the same effect, mimicking hormone or growth-factor interaction with cell-surface receptors, i.e., maintaining growth stimulation.

V. BIOCHEMICAL MEMBRANE ALTERATIONS AND NEOPLASTIC GROWTH

In this presentation we have focused on membrane alterations in preneoplastic and neoplastic cells and tissues, and have tried to discuss their roles in the development of growth advantage and autonomous, deregulated growth. The alterations comprise lipid, protein, and carbohydrate constituents of different membranes. It is not possible, at the moment, to assemble all information into a general functional concept that can explain the mechanisms of tumorigenesis and malignant transformation. We have focused in this article on five areas of particular interest in membrane structure and function and in cellular growth.

Lipid alterations in premalignant lesions seem to be consistent, as reported by several authors. Their impact on membrane stability is limited because alterations in one lipid class are balanced by alterations in other lipids that also affect membrane stability. A reduction in polyunsaturated lipid classes increases the resistance to oxidative stress and lipid peroxidation. In malignant tumors, on the other hand, there are great variabilities in the lipid composition and, at the moment, the picture is too complex to make sense with our present state of knowledge. Alterations in the products of the *mevalonate pathway* in liver nodules are intriguing and seem to support a general growth-competent phenotype, in which growth-limiting supplies of cholesterol and farnesyl are secured. An increase in the level of ubiquinone provides the cell with a potent, inexhaustible antioxidant that probably plays an important role in protecting the cell against noxious agents and processes implying oxidative stress.

Membrane receptors are, with few excep-

tions, downregulated in liver nodules and also in hepatoma. The mechanisms by which the cells retrieve a multitude of different growth-factor receptors from the cell surface are not clear, but most certainly involve alterations in receptor endocytosis, receptor-ligand dissociation in endosomes due to deficient endosomal acidification, and reduced or abolished receptor dimerization or oligomerization. Can receptor alterations, leading to a decreased sensitivity to growth-factor stimulation, be reasonably incorporated into the "resistant" phenotype? It is well known that carcinogenesis does not proceed unless cells are proliferating, either as an effect of carcinogen treatment, chronic inflammation with regeneration, or other proliferative stimuli. Proliferating cells in an environment containing mutagens are more vulnerable to mutagenesis during the replication of DNA than quiescent cells. Hence, in addition to increasing the detoxification and drug elimination, and reducing DNA-adduct formation, part of the rationale for resisting the toxic/mutagenic effect of carcinogens may conceivably also involve a shutdown of externally regulated cell proliferation by downregulation of the relevant growth-stimulatory cell-surface receptor systems. The decrease in signal strength and specificity noted in signal transduction serve the same purpose of making the cell less regulated and more autonomous. The spontaneous growth seen in liver nodules is counterbalanced by increased apoptosis, controlling the size of the population for a long time during the process.

The *multidrug-resistant phenotype* accomplished by altered drug metabolism and drug excretion protects the cell from noxious influences and allows cell growth in the presence of compounds that inhibit the growth of normal cells. The resistant phenotype will thus be of most importance in permitting cell proliferation, at least in the early stages of the process.

Iron deficiency is a consequence of impaired iron uptake. The reduced ability to incorporate iron and to store iron as ferritin complexes might be explained by low endosomal acidification inhibiting endosomal release of iron from endocytosed diferric transferrin and/or decreased reduction of ferric iron on the cell surface and/or in the endosomal vesicles. Iron deficiency increases, by a feedback regulation system, the amount of transferrin receptor sites on the surface of the cells. These receptors help the cell to bring more diferric transferrin to the transplasma membrane NADH diferritransferrin reductase, providing electron acceptors to the cell-surface redox system. Elevated, nonregulated NADH oxidase controls intracellular pH and the NAD/NADH ratio, which are important for the initiation and maintenance of growth. The possible inhibitory effect of cell-surface redox activity on tyrosine phosphatase also contributes to stimulated growth via an increased growth-factor receptor tyrosine phosphorylation. It is, however, too early to speculate further on the mechanisms involved in aberrant signal transduction and its effects on the cell. For the moment, growth-factor receptor tyrosine phosphorylation levels, including kinase and phosphatase regulation, and G-protein heterogeneity, and functions, urgently needs to be investigated.

Liver nodules, putative precursors for malignant liver tumors, exhibit complex phenotypic alterations, many of which will make cells less dependent or less sensitive to environmental regulation and supply. It is obvious from the data presented that drug resistance and resistance to other cellular stress factors are common features in carcinogenesis. The mechanism for this resistance is multifactorial, involving many different genes and complex genetic programs, which seem to be phylogenetically well preserved. The events turning the process on are variable and threatening to the organism. The alterations accomplished by the conducted gene orchestra are beneficial and protect the organism from immediate danger. On rare occasions, imperfections make the program persist, allowing the process to proceed along a discontinuous track to the ultimate malignancy that will kill the organism in the senescence of its life. The objectives for future research are to discover the mechanism to turn on and off the program for the resistance phenotype and the factors that make the concert persist.

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