REVIEW

Are the Phenotypes of Preneoplastic Lesions of Significance for Cancer Prevention? 1. Liver

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Abstract

Preneoplastic lesions have been described for most major sites of human tumour development. They appear to be share characteristics like monoclonality, induction by all classes of carcinogens and some quantitative relationship to actual tumours. Extensive studies of preneoplasia in the liver of the rat has indicated that a directed shift in phenotype occurs, commensurate with greater physiological emphasis on growth potential. One characteristic change is increase in the key enzyme of the pentose phosphate pathway, glucose 6 phosphatase dehydrogenase as well as elevation in glycolysis, reduction of gluconeogenesis. In general, the changes observed in preneoplastic liver lesions appear reminiscent of the effects of insulin or other hormones on hepatocytes, pointing to possible application of specific inhibitors for cancer chemoprevention.

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Introduction

The prevailing paradigm in cancer research is that malignancies arise from preneoplastic focal lesions by accretion of increasing numbers of genetic alterations, the processes underlying neoplastic development being termed initiation, promotion (or modulation) and progression. The latter, characterized by genetic instability, results in a marked biological complexity which is reflected in immense difficulties in therapeutic control. It is to be hoped that advances in molecular understanding will eventually generate new approaches to surmount this problem but, as an adjunctive strategy, it is clear that preventive efforts must also be emphasized. One question of importance is therefore what can the phenotypes of preneoplastic lesions tell us regarding how best to counter their development (primary prevention) or progression towards malignancy (secondary prevention).

In experimental efforts to develop of concepts as to how neoplasia occurs, the liver has played a central role, and a great deal of information has been obtained regarding the characteristics of different stages in tumour development (Bannasch, 1996; Farber 1985; Pitot, 1996). In terms of the biochemical and biological characteristics, focal lesions occurring in the liver of the rat have attracted particular interest. Details for other species and organs are relatively scarce although there is evidence that common shifts in phenotype may occur in preneoplasia independent of the organ. Certainly there are essential prerequisites for classification of preneoplastic lesions, like induction by carcinogens and some quantitative relationship to tumour development (Ogiso et al., 1990), but reversibility and heterogeneity in potential for malignant conversion complicate these issues.

Clonal Origin from Single Cells

The earliest change that can be discerned in the livers of rats administered a genotoxic chemical carcinogen, other than those due to toxicity, is the single glutathione-S transferase positive hepatocyte. These putative 'initiated' cells show a time and carcinogen dose-dependence, and their numbers can be modulated by induction of drug metabolizing enzymes (Moore et al., 1988). They may arise preferentially in the mid-zone in the liver although this has been disputed (Kato et al., 1993). Whatever, the evidence of a clonal, but multiple origin for foci, with multiple lineages for tumors, is in line with findings from studies of expression of X-chromosome-linked genes in chimaeric mouse foci (Rabes et al., 1982) and tumors (Williams et al., 1983). Similar results have also been gained for γ-glutamyl transpeptidase positive foci in mosaic mice (Tsuji et al., 1988). Furthermore, evidence has been documented of a clonal origin for human adenomatous hyperplasias (Tsuda et al., 1988) and from studies of hepatitis virus integration in carcinomas (Esumi et al., 1986).

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Morphological Alteration in Preneoplastic Foci in the Liver

Whether they appear 'spontaneously' (Schulte-Hermann et al., 1983) or are caused by chemical carcinogens (Farber, 1956; Bannasch and Zerban, 1992; Pitot, 1990), X-rays (Kitagawa et al., 1985; Maisin et al., 1993), α-particles of Thorotrast and neutrons (Ober et al., 1994), or viruses (Toshkov et al., 1990), the majority of lesions share basic similarities in their morphological appearance and a number of biochemical characteristics. These depend to some extent on the conditions under which they arise but generally they initially demonstrate clear cell character, reflecting excess storage of glycogen (see Figure 1) (Bannasch 1996). Closely related are the acidophilic or 'eosinophilic' lesions (Squire and Levitt, 1975) which show pronounced proliferation of smooth endoplasmic reticulum as well as increased glycogen. The histogenesis most often involved in generation of benign adenomas and invasive carcinomas from foci under various experimental conditions has been described previously in great detail (Moore et al., 1982; Weber and Bannasch, 1994a;1994b; 1994c). The typical sequence leads from glycogen storing foci (GSF) to mixed cell foci (MCF), which grow to take on supra-acinar proportions, compressing the parenchyma as nodules, and may give rise to intensely

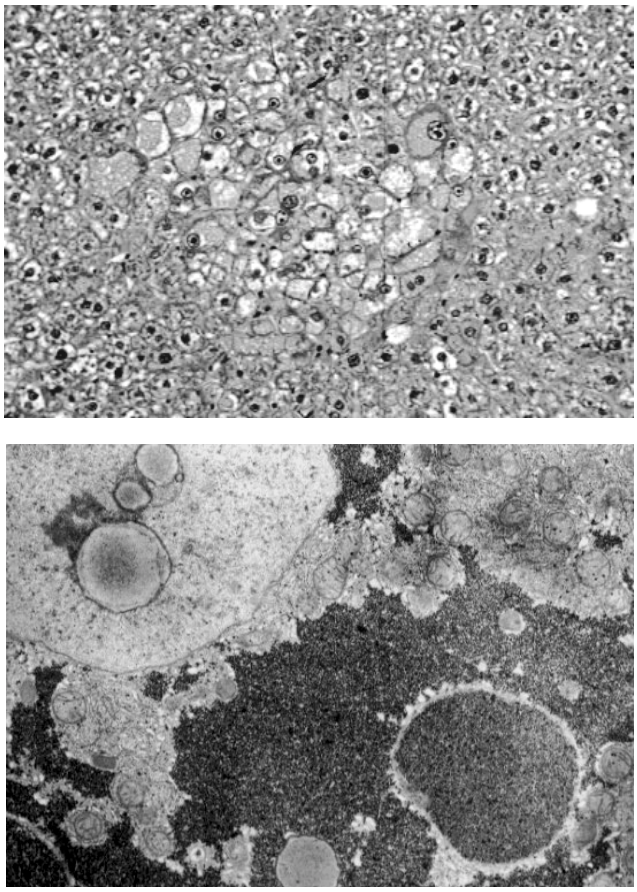

Figure 1. a) Glycogen Storage Focus, LM b) Hepatocyte within a Glycogen Storage Focus, EM

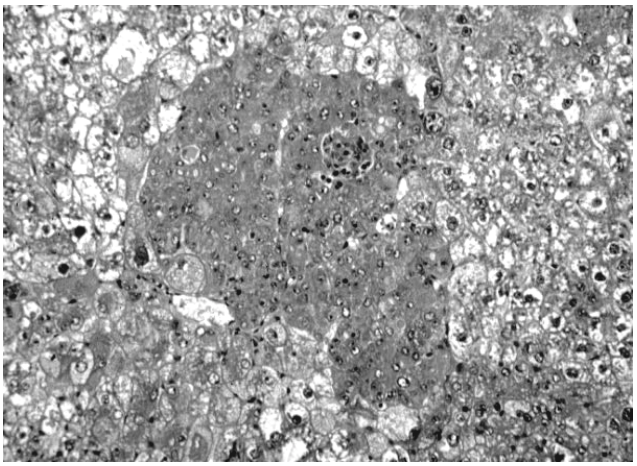

Figure 2. Basophilic Focus within an MCF

basophilic populations of dysplastic cells, similar to those characteristic of malignant tumors, observed within their masses (see Fig 2) (Moore et al., 1982). The same basic elements have now been documented for preneoplastic and neoplastic liver lesions induced by carcinogens in other rodents (Bannasch et al., 1979; Pitot, 1990; Ungar and Adler, 1978), chickens (Brunn et al., 1987) and the rhesus monkey (Ruebner et al., 1976), as well as in rats by irradiation (Ober et al., 1994), in transgenic mice (Toshkov et al., 1994) and in the woodchuck infected with woodchuck hepatitis virus (Toshkov et al., 1990). Glycogen storage in phenotypically altered foci has also been described for the human liver in (Fischer et al., 1986; Karhunen and Pentilla, 1987; Su et al., 1997). Somewhat exceptional are the foci which arise in association with long term application of so-called non-genotoxic or epigenetic carcinogens, including the peroxisomal proliferator-class of agents . Variously termed 'atypical eosinophilic foci' (Harada et al., 1989), 'homogeneous basophilic foci' (Marsman and Popp, 1994) or as having weak diffuse basophilia (Kraupp-Grasl et al., 1990), these are poor in or free of glycogen, and due to their combination of intense acidophilia and randomly scattered or diffuse basophilia have been termed 'amphophilic foci' (APF) (Weber et al., 1988a). There is evidence that they progress to adenomas with similar characteristics and highly differentiated carcinomas (Weber et al., 1988b).

Altered foci in the liver have been well characterized in terms of their enzyme phenotype (see Fig 3), with a directed in shift in different individual species observed with increase in size or progression towards nodules (Tsuda et al., 1992; Yamaguchi et al., 1993). Physiological significance can best be assessed in terms of key enzymes of metabolic pathways.

Enzyme Phenotypic Alteration in Preneoplastic Foci in the Liver

a) Carbohydrate and energy metabolism.

Glycogen storage and breakdown and glucose uptake and

release play a pivotal role in regulation of blood glucose and foci can be distinguished from neighbouring cells by a pattern of elevated and decreased enzyme activities. At the interface between the cell and the blood a facilitative transport mechanism operates in the liver, involving primarily the low-affinity type GLUT 2 transport protein. This is decreased in foci and nodules (Grobholz et al., 1993; Mayer et al., 1995), with the appearance of GLUT 1 expression beginning in late MCF, and increasingly thereafter, in the majority of adenomas and carcinomas, as well as in microvessels adjacent to adenoid formations in tumors (Grobholz et al., 1993). The results point to an initial decrease of glucose transport so that only small amounts enter and perhaps more importantly leave the foci. This is in line with the observed reduction in G6Pase (Hacker et al., 1982). Since the Km value is very high it is only under high glucose concentrations that this step would become limiting. In terms of hormonal control it is well documented that GLUT 2 expression is decreased by insulin (Andersen et al., 1994; Burcelin et al., 1992). The expression of GLUT 2 in APF remains to be determined.

The increase in GLUT 1 observed for rat nodules (Grobholz et al., 1993) is in line with earlier findings for human HCCs (Su et al., 1990). It might indicate a high glucose consumption, as also suggested by the reported shift from glucokinase to hexokinase in human tumors (Hammond and Balinsky, 1978). Increase in hexokinase activity would not be expected to be inhibited by the product due to mitochondrial binding (Arora et al., 1992). Judging from immunohistochemical findings for some experimental preneoplastic lesions this might also be the case for foci (Fischer et al., 1987). However, using a laser microdissection and microbiochemical approach, an increase in hexokinase could be shown in HCC but not GSF or MCF (Klimek and Bannasch, 1993). Histochemically, hexokinase/ glucokinase activities have been shown to be normal or increased in GSF and generally unchanged in APF (Metzger et al., 1995).

The metabolic flow from glucose to glucose-6-phosphate is under pancreatic hormone control, with insulin causing increase in the glycolytic direction and glucagon acting to promote glucose release. In these terms, GSF can be regarded as having escaped from the predominantly gluconeogenotic function of the normal hepatocyte in the periportal and intermediate zones of the liver. This is supported by the finding of glycogen retention in foci in the early stages of fasting (Bannasch et al., 1984) although this might be just a question of absolute amounts of substrate available for degradation. APF, on the other hand, with their generally elevated G-6Pase activity might be expected to have relatively low levels of intracellular glucose. It is not clear how they behave under conditions of restricted calorific intake.

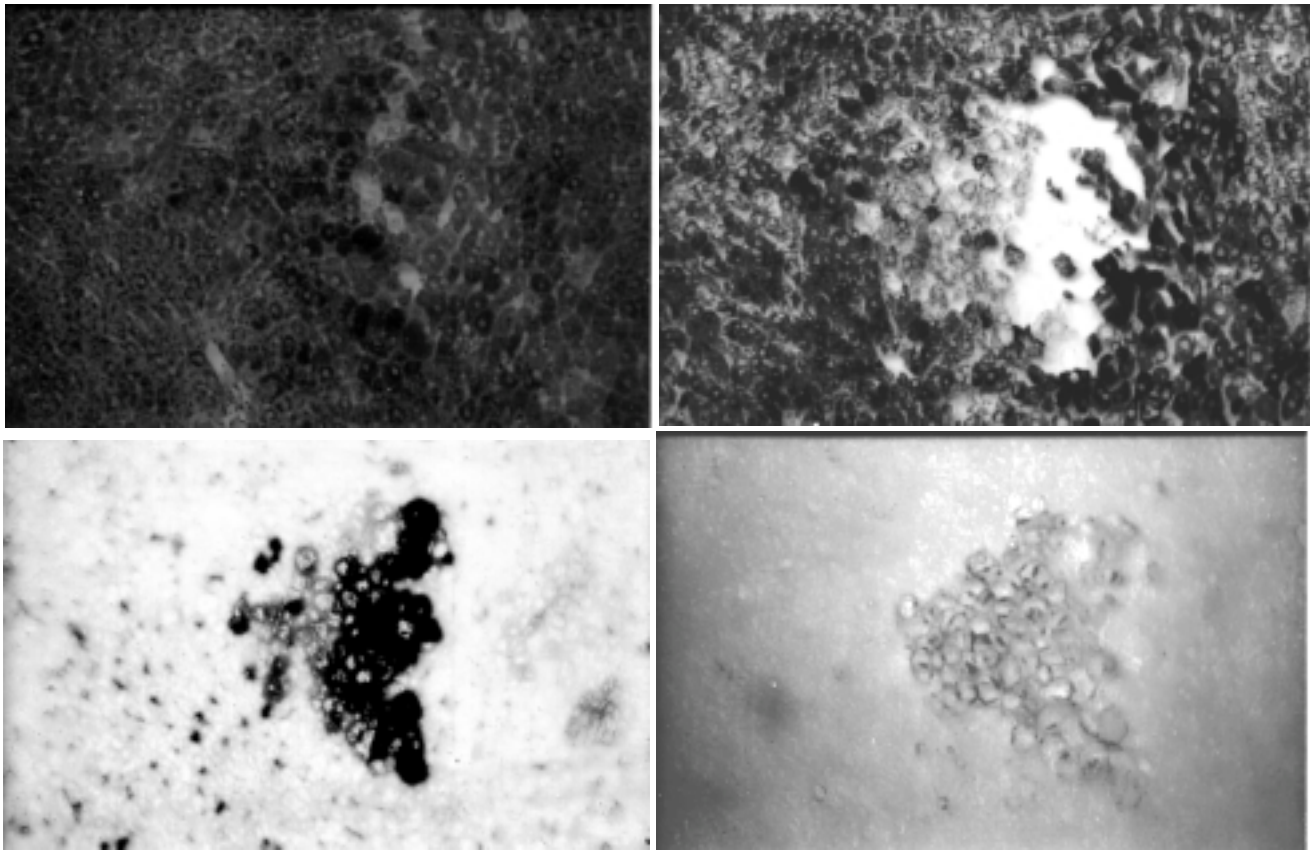

Figure 3. Representative Histochemical Results for an MCF in Rat Liver. Semi-serial Sections Reacted for: a) PAS-Alcian Blue; b) G6Pase; c) G6PDH; d) γGT

With regard to the pronounced storage of glycogen evident in GSF, it is still unclear what is the principal underlying biochemical alteration. The metabolite G6P has been established as a control point with regard to glycogen storage in an in vitro cell line demonstrating many of the characteristics of GSF (Mayer and Letsch,1989) and its elevation has been described in rat livers after treatment with nitrosomorpholine (Enzmann et al., 1988). However, absolute levels within foci are very difficult to measure in vivo. As would be expected from the increase in glycogen stores, the activity of the synthetic enzyme glycogen synthase is increased, whereas that for breakdown, the phosphorylase, is more variable but most frequently decreased (Hacker et al., 1982; Metzger et al., 1995). This has also been confirmed at the protein level by immunohistochemistry (Seelmann-Eggebert et al., 1987). Lysosomal α-glucosidase activity is also decreased in GSF while becoming increased in MCF (Klimek and Bannasch, 1989). The available data in fact point to a decrease in glycogen breakdown and not increased synthesis as the most important factor responsible for excess storage. In MCF, the glycogen decrease appears due to both a drop in synthase activity (Hacker et al., 1982) and the increase in α-glucosidase (Klimek and Bannasch, 1989).

The question of the relative importance of different sources of G6P for glycogen synthesis remains unclear. The marked concentration differential across the cell membrane from the serum to the cytoplasm would presumably mean that even under conditions of reduced GLUT 2 the foci still obtain an appreciable glucose supply from the blood. The later GLUT 1 increase would supply an elevated inward flow but this can not explain the increased glycogen storage in early lesions since it generally occurs in more basophilic populations. It is possible that the cells might express other glucose transporters, such as GLUT 3 which is known to be expressed in some liver tumors (Yamamoto et al., 1990), but this would appear unlikely. Another possibility is that other precursors like amino acids or lactate can be channeled via gluconeogenesis (Baggeto, 1992), but it is unlikely that key enzymes in this pathway are increased in GSF given the stimulated state of glycolysis (Hacker et al., 1982; Klimek and Bannasch, 1990). The involvement of insulin and glucagon in glycogen metabolism has been the subject of almost innumerable investigations and the excess in GSF and relative lack in APF clearly suggest very different biochemical conditions indicative of alteration in hormone dependent pathways. Furthermore, liver amyloglucosidase can be altered by starvation and insulin treatment (Skoglund et al., 1987). It should also be mentioned here that a number of enzymes involved in biosynthesis of glycosaminoglycans are increased and their degradative counterparts are decreased in the livers of rabbits by administration of insulin (Padmaja et al., 1978).

In GSF and to a larger extent in MCF, there is a consistent increase in the activity of the key enzyme of the pentose phosphate pathway, G6PD (Butler et al., 1981; Hacker et al., 1982; Moore et al., 1983). Confirmed by microbiochemical analysis of microdissected tissue (Klimek et al., 1984) and immunohistochemical staining (Moore et al., 1987) this has been shown to be at least partly due to elevated expression of mRNA (Stumpf and Bannasch, 1994). It may be associated with elevated proliferation through provision of ribose-5-phosphate units (Baba et al., 1989) and in this sense the histochemical findings fit with the fact that cell division is known to be stepwise increased in GSF and MCF (Zerban et al., 1989). However, the correlation with BrdU incorporation is not perfect at the individual cell level in foci (Moore et al., 1987). APF in contrast have a high proliferation rate (Marsman and Popp, 1994) without any elevation in G6PDH (Metzger et al., 1995). With regard to hormonal control of G6PDH in cultured rat hepatocytes, insulin is well known to induce mRNA synthesis (Manos et al., 1991). With fetal hepatocytes, insulin increases and glucagon decreases mRNA levels (Molero et al., 1994).

The key enzymes of glycolysis are GAPDH, not changed in GSF but generally increased in MCF (Hacker et al., 1982), and pyruvate kinase (PK), which demonstrates increase in GSF as assayed histochemically and biochemically (Klimek and Bannasch, 1990). While PK is decreased in more advanced macronodules induced by thioacetamide (Klimek et al., 1988), and also in Solt-Farber nodules (Gerbracht et al., 1993), reflecting a reduction in L-type protein (Fischer et al., 1987), it has been shown to be increased in many GSF by Bannasch and co-workers. It has also been established that APF are usually normal or decreased for PK (Metzger et al., 1995), which is upregulated by insulin and downregulated by glucagon (Granner and Pilkis, 1990; Munnich et al., 1984). However, the data suggesting a negative correlation between APF and glycolysis are not consistent and an earlier publication documented increase in GAPDH activity in some APF (Weber et al., 1988).

b) Amino acid and protein metabolism

Turning to the theme of protein synthesis and energy production, again we are hampered by the very limited nature of the information on the activity of mitochondrial enzymes in different types of foci which has so far been published. In one study of N-ethyl-N-hydroxyethylnitrosamine-induced foci, succinate dehydrogenase was found to be decreased while isocitrate DH and malate dehydrogenase were increased (Moore et al., 1986). Similar decrease in SDH has also been described for a number of other cases (Butler et al., 1981; Kitagawa, 1971; Metzger et al., 1995), although its significance is unclear since it is not considered to be a rate limiting enzyme. It appears, however, to be increased in a majority of APF (Metzger et al., 1995) and it should therefore be mentioned that it can be induced by glucagon (Mohan et al., 1991). Stimulation of ICDH, along with oxaloacetate dehydrogenase and pyruvate dehydrogenase (PDH), is associated with increase in Ca++ (Denton et al., 1987). The increase in GDH which has been reported for GSF and glycogen storing nodules (Moore et al., 1986; Metzger et al., 1995) and its decrease in APF (Metzger et

al., 1995) might be interpreted as indicating differences in protein synthesis, insulin stimulating incorporation of Krebs cycle derived glutamate into proteins (Mohan et al., 1991). Glucagon on the other hand favours partitioning of pyruvate towards carboxylation, by increasing the flux through pyruvate carboxylase, apparently without directly inhibiting that through PDH (Agius and Alberti, 1985).

With regard to protein metabolism, again, the data are very limited. However, amino acid catabolism may be reduced in GSF, as evidenced by decreased serine dehydratase (Kitagawa and Pitot, 1975) and a lack of inducibility of tryptophan oxygenase (Moore et al., 1986). Transcription of mRNA for both of these enzymes is induced by decreased feeding states and inhibited by insulin (Kanamoto et al., 1991; Nakamura et al., 1987). The findings for acid phosphatase and β-glucuronidase (Kitagawa, 1971; 1976) with a clear reduction in activity within GSF might also be interpreted as indicating that they have a reduced capacity for proteolysis, as described earlier for liver nodules in the Solt Farber model (Ahlberg et al., 1987). APF exhibit increased breakdown capacity, in line with their relatively small cell size (Metzger etal., 1995), since a decrease in the latter is associated with alkalisation of the acid intravacuolar pH which is necessary for the effective action of breakdown enzymes (Haussinger et al., 1994). This might also be of importance for the regulation of α-glucosidase. Glucagon is known to induce biliary protein excretion in Guinea pigs (Lenzen and Tavolini, 1993), accounted for primarily by an increase in the lysosomal enzymes acid phophatase and β-glucuronidase. Elevated lysosomal pH may also be responsible for the loss of iron storage capacity observed in most foci (Williams et al., 1976), as argued by Eriksson et al (1986). It is interesting in this context that basophilic foci which spontaneously arise in F344 rats do not exclude iron (Stitzel et al., 1990).

Decreased protein degradation, suggestive of an anabolic metabolism, is generally in agreement with findings for RNase (Daoust, 1979) and nuclease (Fontaniere and Daoust, 1973; Taper and Bannasch, 1976) within GSF and MCF.

b) Lipid metabolism and membrane biochemistry

The key metabolite in lipid metabolism is acetyl CoA (see Fig 4). Unfortunately, the enzymes which are primarily responsible for directing its utilization into different pathways are not accessible to investigation by histochemical methods. Therefore, most information which has so far been published in this area has been gained using the Solt-Farber nodules which are large enough or occupy sufficient parenchyma for standard biochemical tests to be applied. The group of Eriksson has been particularly active in this area (Eriksson and Andersson, 1992).

Lipid droplets can be stained with Oil Red O but results have been very heterogeneous, in some cases foci or nodules being found to lack any indication of lipid synthesis (Moore et al., 1986) and in others a marked increase leading to the

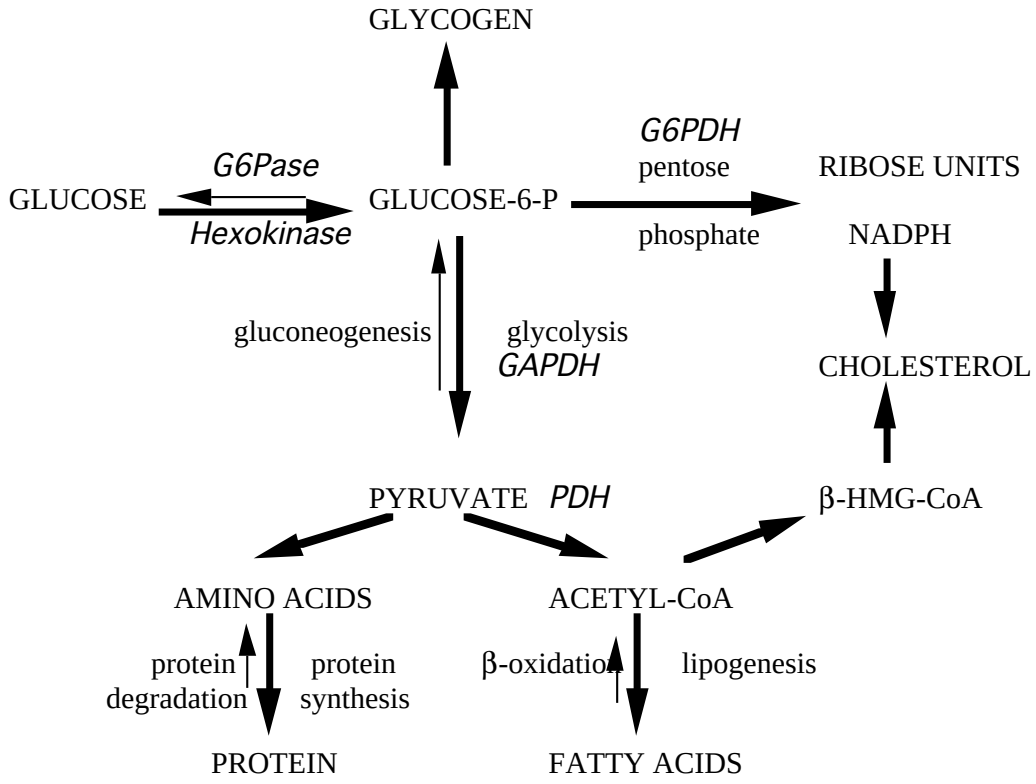

Figure 4. General Anabolic Phenotype of GSF/MCF in the Rat Liver

appearance of fat storing foci have been described (Bannasch et al., 1972). In this latter case it is often correlated with a transition to more basophilic populations,. It is also seen in lesions in woodchucks infected with hepatitis B virus and receiving aflatoxin (Bannasch et al., 1995). Data for the key enzymes of lipid synthesis have not been published for foci, but it is well known that they are induced by insulin or a high glucose diet and suppressed by glucagon, starvation or diabetes (Noguchi et al., 1992). Interestingly, fructose, a hepatopromoter causing similar enzyme changes in liver parenchyma to those observed in foci (Enzmann et al., 1989) induces hypertrigyceridaemia through activation of pyruvate dehydrogenase and synthesis of new fat (Park et al., 1992). The malic enzyme which is often cited as being primarily responsible, along with G6PDH, for supplying reduced NADP for lipogenesis is consistently increased in GSF and MCF and may be reduced in APF (Metzger et al., 1995). Malic enzyme is post-transcriptionally increased by insulin in the normal rat liver (Davis et al., 1988), but can also be induced at the mRNA level (Garcia-Jimenez et al., 1994). Glucagon on the other hand appears to primarily reduce malic enzyme in a post-transcriptional fashion (Goodridge et al., 1986). However, functional interpretation of changes in malic enzyme activity are made difficult by the number of metabolic pathways which are dependent on NADPH.

The pathway opposite to that of lipid synthesis, the β-oxidation of fatty acids which occurs in mitochondria and in peroxisomes for very long chain species, has been reported to be reduced or non-inducible in both GSF and APF (Yokoyama et al., 1992;1993), although in the latter case individual lesions may demonstrate considerable heterogeneity in terms of peroxisomal enzymes (Grasl-Kraupp et al., 1993a). In human liver biopsy specimens, glucagon enhances oxidation of fatty acids with subsequent production of ketone bodies (Vons et al., 1991). Peroxisomal beta-oxidation enzymes may be negatively regulated by insulin (Sorensen et al., 1992), in line with the control of peroxisomal proliferator-activated receptors (Steineger et al., 1994).

With regard to the metabolism of acetyl CoA to ketone bodies, it has been reported that GSF and glycogenotic nodules have a decreased β-hydroxybutyrate dehydrogenase (Moore et al., 1986). Whether this reflects altered production or use as fuel, it is of interest here that, in the starved rat, insulin decreases production of 3-hydroxybutyrate and glucagon causes an increase (Reed et al., 1984).

Given the importance of cholesterol for growth of carcinogen-induced lesions (Ledda-Columbano et al., 1985) the mevalonate and associated pathways are of especial importance. In the rat liver, foci and nodules have been variously described as having normal or reduced cholesterol, while dolichol, another lipid product of the mevalonate pathway is increased, along with ubiquinone (Olsson et al., 1991; 1995). These lipids have effects on the physico-chemical properties of membranes, determining fluidity for example. The key enzyme of the mevalonate pathway, β-hydroxy-β-methylglutarate-coenzyme A (HMG Co A) reductase, is increased in nodules induced by different models (Olsson et al., 1995; Coni et al., 1992), associated with hypomethylation of the gene (Coni et al., 1992). This enzyme also increased in regenerating liver (Trentalance et al., 1984). Insulin stimulation and glucagon dependence of HMG CoA reductase is controlled at the mRNA and protein levels (Ness et al., 1994). Inhibition by cAMP has also been documented (Botham, 1992). However, while an increased capacity for cholesterol synthesis has been shown in nodules (Ledda-Columbano et al., 1985) other authors have noted a lack of increase in cholesterol levels or squalene synthase activity (Olsson et al., 1995). The question of whether this is of over-riding significance in determining flux through the pathway remains unclear, however, and rapid incorporation of cholesterol into membranes might explain why no increase is observed. Cholesterylester levels are doubled in some membrane preparations in nodules (Olsson et al., 1991) and membrane cholesterol may influence B-adrenergic receptor characteristics and adenylate cyclase (Scarpace et al., 1985). The enzyme is controlled by several feed-back regulatory pathways including cholesterol and plasma LDL (Goldstein and Brown, 1990) and reduction in cell surface receptors for LDL has been reported in nodules (Harris et al., 1987).

However, rapid uptake of dietary cholesterol nevertheless may occur (Horton et al., 1973) and the possibility that the reductase gene is resistant to normal feed-back control due to hypomethylation must also be taken into account (Coni et al., 1992). Interestingly, the hormone DHEA causes decrease in HMGCoA reductase while increasing LDL receptor expression (Pascale et al., 1995).

Another potentially important feature of foci and nodules may be that the isoprenylation of regulating proteins may be elevated. Thus both farnesyl PP synthase and all trans geranylgeranyl PP synthase, respectively responsible for production of farnesyl and geranyl geranyl units, demonstrate increased activity in nodules (Olsson et al., 1995). The importance of this can be judged from the fact that the GTP-binding and GTP-hydrolyzing p21ras is activated by covalent farnesylation of the cysteine four residues form the COOH terminus of the peptide, this leading through a cleavage reaction to allow binding to the inner surface of the plasma membrane (Maltese, 1990). Inhibition of mevalonate synthesis by HMG-CoA inhibitors causes accumulation of unfarnesylated forms (Repko and Maltese, 1989) and blockage of growth (Goldstein and Brown, 1990). It is unclear at present whether APF also demonstrate alteration in this pathway but it is of interest that DHEA, an adrenal steroid inducing such lesions, has been reported to inhibit protein farnesyltransferase (Schulz and Nyce, 1994). Other proteins which undergo activation through isoprenylation include members of the rho, rap, rac, ral, and rab families as well as nuclear lamins (Maltese, 1990).

It has been shown that N-acetylglucosaminyltransferase, an enzyme responsible for insertion of a bisecting N-acetylglucosamine (bi-Gn) into complex-type N-linked glycans of cellular proteins, is induced in liver nodules and

timors induced by a number of regimens in rat (Pascale et al., 1989). Dolichol synthesis, which is low in diabetic rats, returns to normal with application of insulin (Sharma et al., 1987). The observed increase in ubiquinone (Olsson et al., 1995) could be of importance for lipid peroxidation, since it may act as a natural lipoprotein antioxidant in its reduced form (Eriksson and Andersson, 1992). Ubiquinone, or coenzyme Q functions mainly as a redox component in mitochondria but may also act as a membane and lipoprotein antioxidant in its reduced form (Kagan et al., 1990). Its elevated level in foci and nodules could therefore contribute to resistance to toxicity and also the reported failure to generate lipid peroxidation products in response to a prooxidant stimulus (Poli et al., 1986).

Regarding breakdown of cholesterol to bile acids, the situation in foci and nodules remains to be explored but it should be noted that the key enzyme cholesterol 7 alpha-hydroxylase is inhibited by insulin (Twisk et al., 1995). Glucagon, in contrast, increases internal secretion of cholesterol and transformation into bile acids (Guettet et al., 1988). This process appears to be modulated by cAMP-dependent protein kinase (Tang and Chiang, 1986)

c) Cell surface, physical characteristics

The membrane receptor system has three components: extracellular ligand, membrane receptors and the intracellular messenger and cycling system.

As noted above, the LDL receptor may be decreased in rat liver nodules (Harris et al., 1987) and this is also reported to be the case for the asialoglycoprotein receptor (Andersson et al., 1988). EGF-R mRNA levels reduced in nodules (cited in Eriksson and Andersson, 1992) and after partial hepatectomy (Johansson et al., 1990). In the latter case both high and low-affinity sites affected wheras in nodules decrease limited to high-affinity sites (Rissler et al., 1991). Increase has however also been described in nodules, along with TGFa in correlation with PCNA assessed proliferation (Tanno and Ogawa, 1994). Transgenic mice with TGFa have increased HCC (Takagi et al., 1993). Also increased TGFA during rat hepatocarcinogenesis (Kaufmann et al., 1992; Perez-Tomas et al., 1992). Location of TGFa of interest, cytoplasmic in persistent nodules (Tanno and Ogawa, 1994), cell membrane in TGFa transgenic mouse lesions (Takagi etal., 1993). Membranous pattern also in regenerating hepatocytes (Burr et al.,1993). Increased EGF-R in human HCCs (Fukusato et al.,1990) but not DEN-induced lesions in rats (Hsieh et al., 1987). Correlation between GH-R and EGF-R m RNA levels has been noted in regenerating liver, liver nodules and hepatoma (Levinovitz et al., 1990). Hepatic production of IGF-I is controlled by GH in the liver and thus the reduced expression found in both liver nodules and HCCs (Norstedt et al., 1988) is in line with expectations on this score. Large fraction of nodular GH-R carry bound GH (Levinovitz et al., 1990), explanation for the fact that nodules no longer demonstrate normal sexual differentiation (Blanck et al., 1990). GH-R also decreased after PH but difference from nodules in that endocytosed ligand-receptor is rapidly degraded (Eriksson and Anderssen, 1992). In nodular cells in the Solt Farber model, similar resistance to inhibition of EGF-stimulated DNA synthesis by TGF-β as found in normal hepatocytes (Wollenberg et al., 1987). TGF-β is produced in non-parenchymal cells (Nakatukasa et al., 1990) and therefore here is a possible influence of Kupffer cells. A reduction of these has been described in some but not all models (Janossy et al., 1986; Mayer et al., 1995; Ogawa et al., 1983).

From examination of the increased nodular expression of transferrin receptors (Eriksson et al., 1986) and decreased asialoglycoprotein receptors, it has been surmised that an altered capacity for endocytic processing might be playing a role in nodules (Eriksson and Andersson, 1992). Vacuolar pH of nodular cells is elevated as evidenced by decreased activity of the vacuolar-type H+-ATPase and quenching of acridine orange (Andersson et al., 1989). Nodules exhibit reduced proteolysis and a deficiency of certain hydrolytic enzymes (Ahlberg etal., 1987) as noted above. Decreased vacuolar acidification may be a mode of downregulating the responsiveness to growth regulation (Eriksson and Andersson, 1992). Interesting in this respect is the alkalinization of lysosomes by ras transformation of fibroblasts (Jiang et al., 1990) and by factors causing increased cell volume (Busch et al., 1994).

This change in lysosomal pH may be related to the observed decreased ability to store iron in FAH (Williams et al., 1976), and decreased levels of heme-containing enzymes such as cytochrome P-450, cytochrome b5, and tryptophan 2,3-dioxygenase as well as cellular heme and heme-binding protein (Farber et al., 1989). Increased heme oxidase, responsible for degrading heme, and decreased heme-synthetizing activity expressed as activity of 5-aminolevulinic acid synthase have also been reported (Roomi et al., 1985; Stout and Becker, 1986; 1987).

Fibronectin binding protein AGp110 is reduced in aflatoxin induced foci and nodules, to a lesser extent also fibronectin and a5ß1. Acinar structures in tumors demonstrated pericellular and luminar staining, and the fact of a total loss in poorly differentiated tumors (Stamatoglou et al., 1991) suggests that this is a reflection of altered differentiation.

Gap junctional intercellular communication is known to play an important role in maintaining physiological hoeostasis under normal conditions by mediating transfer of signal transducing substances such as calcium, cyclic AMP and inositol triphosphate responsible for physiological control of the cell cycle and growth (Saez et al., 1989). Its interruption would therefore be expected to exert a major influence on growth control and both foci and nodules show obvious decreases in levels of mRNA and immunohistochemically demonstrable connexin 32 (Fitzgerald et al., 1989; Krutovskikh et al., 1991). Cell proliferation in focal lesions as well as after partial hepatectomy is correlated with the decrease in C32 (Tsuda et al., 1995) and CCl4 toxicity brings about a reversible block in gap junctional function (Saez et al., 1987). Regarding the

regulation of cell-cell communication it is apparent that phosphorylation status is of essential importance and a number of authors have reported involvement of both cAMP-dependent protein kinase and protein kinase C (Godwin et al., 1993; Stagg and Fletcher, 1990). Generally the insulin associated PKC reduces expression of connexins and interfers with communication, while the glucagon-stimulated PKA exerts a reverse effect. However, regulation by these protein kinases is in a complex, interrelated manner, presumably by multiple phosphorylation of proteins within gap junctions. Individual gap junction proteins can also be independently regulated, as shown for connexins 26 and 32 during hepatocarcinogenesis (Sakamoto et al., 1992).

d) Drug metabolism

The resistance of FAH to the toxic and mitoinhibitory effects of carcinogens has long been a focus of interest (Farber et al., 1976) and indeed is the basis of the short-term "selection pressure" model, initially conceived by Haddow (1938) and developed by Farber and his colleagues (Solt and Farber, 1976; Solt et al., 1977). It appears to be due to specific alteration in phase I and II drug metabolizing enzymes (Roomi et al., 1985) and is reflected in reduction of adduct formation by DMN, for example (Ozaki et al., 1993) or AAF (Huitfeldt et al., 1986). Foci are esistant to the necrogenic effects of galactosamine (Laconi et al., 1992) and CCl4 (Tsuda et al., 1987).

Regarding phase I species, both P450 I and II are generally decreased in rat liver foci (Buchmann et al., 1985; Cameron et al., 1976; Tsuda et al., 1987; 1988) and the mono-oxygenase sysem is downregulated in mouse tumours (Becker and Stout 1984). Similar characteristics are shared by human tumors (El Mouehli et al., 1987).

In the APF results for P450IV variable, some show increase and others decrease (Grasl-Kraupp et al., 1993a). Because of the heme core iron deficiency could be partly responsible, but this is unlikely given the variation in relative expression of duifferent members of the cytochrome P 450 gene family. Furthermore, no decrease in P450, despite reduction in AHH, with long-term iron deficiency (Rao and Jagadeesan, 1995). Therefore some other control mechanism in play, known to be downregulated in human hepatocytes in primary culture by cytokines (Abdel-Razzak, et al., 1993). Glucagon can strongly induce some P-450 forms, although the total remains unchanged (Rouer and Leroux, 1985). Kinases are very specific for phase I and II enzymes (Pyerin et al., 1987) and there may be increases the intracellular degradation of specific forms (Eliasson et al., 1992). Phosphorylation of P450 activates the enzyme and thereby brings about elevated lipid peroxidation (Mkrtchian and Andersson, 1990). Potential links between the mixed function oxidase system and enzymatic glucose metabolism have also been stressed (Karvonen et al., 1987).

With regard to phase II detoxification a number of parameters have been investigated in GSF and MCF type lesions, with a particular focus on glutathione metabolism. Thus reduced glutathione itself is apparently increased (Ahluwalia and Farber, 1984; Deml and Oesterle, 1980), along with glutathione peroxidase and reductase (Kitahara et al., 1983) and many of the glutathione S-transferase family (Kitahara et al., 1984; Tatematsu et al., 1985) including GST-P in particular (Sato et al., 1985) (see Fig 5). This might be related to the overexpression of P glycoprotein and multidrug resistance in nodules (Fairchild et al., 1987; Thorgeirsson et al., 1987). APF, in contrast demonstrate a decrease in GST isoenzymes (Grasl-Kraupp et al., 1993b). The frequent GSF/MCF increase in the related enzyme GGT is also not shared by amphophilic cells (Rao et al., 1986; Metzger et al., 1995). Hepatic gamma-glutamylcysteine synthetase and glutathione synthesis in the rat is dependent on insulin as well as glucocorticoid (Lu et al., 1992). Positive in vivo regulation of glutathione S-transferase by serum insulin has also been reported (Carnovale et al., 1990) and insulin given together

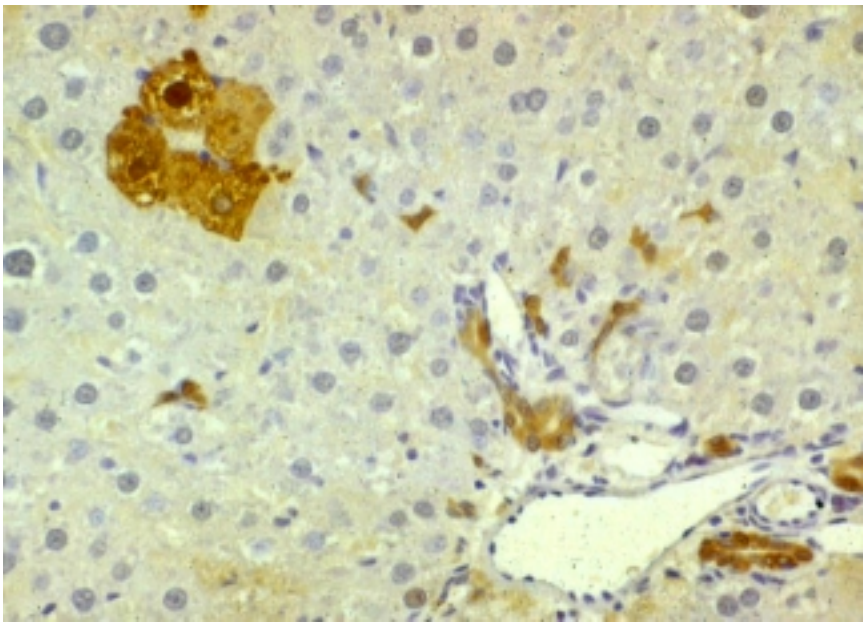

Figure 5. Immunohistochemical Staining of GST-P in Rat Liver. Note the strong expression in a mini-focus and also the epithelial cells of the bile ducts, both proliferating under Solt-Farber conditions.

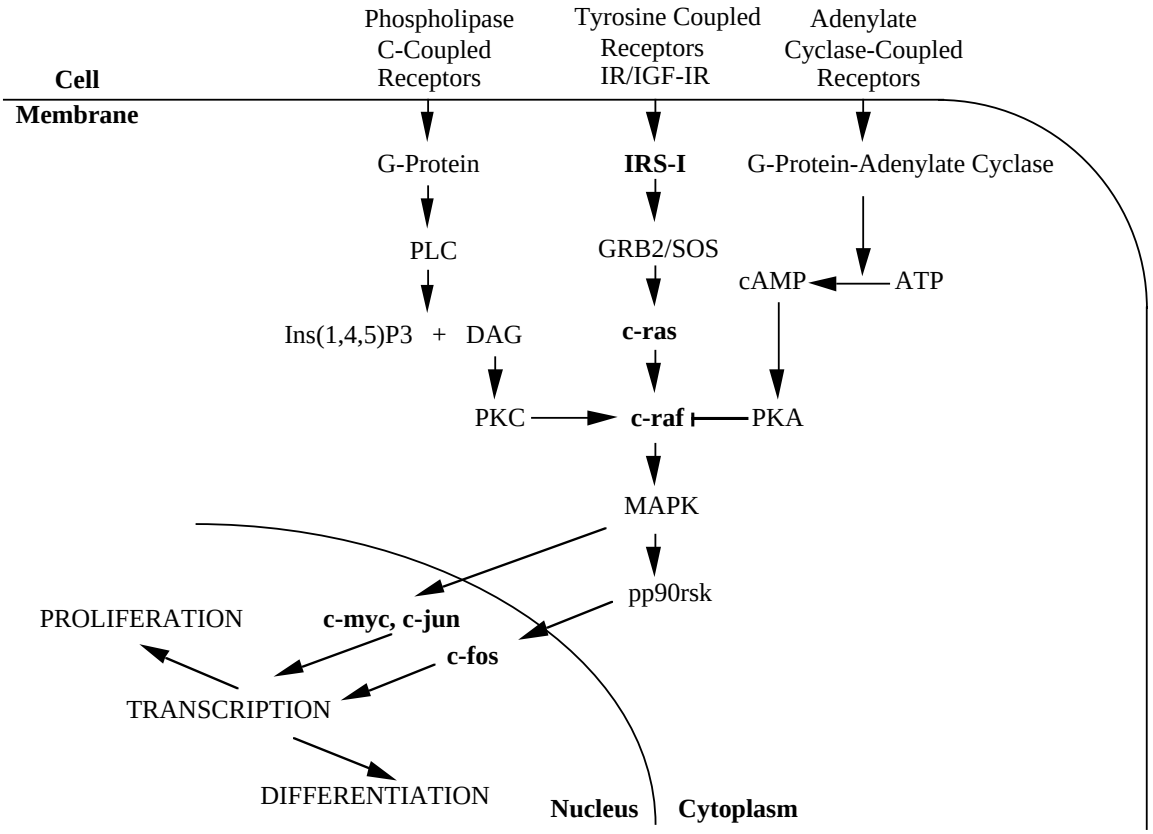

Figure 6. Signalling Pathways. Note the components for which incease has been reported in foci and/or nodules (bold)

with epidermal growth factor induces GST-P in adult rat hepatocytes in culture (Hatayama et al., 1991). Acute upregulation of hepatic cytosolic glutathione S-transferase activity by iv administration if insulin has also been reported (Carrillo et al., 1995). This is in line with the finding that NIDDM is associated with lowered glutathione levels and reduced GST activity (Barnett et al., 1992). Diabetes is also associated with decreased liver catalase, glutathione peroxidase and superoxide dismutase as well as GSH, which is reversed by insulin treatment (Wohaieb and Godin, 1987). The fact that some GST species are good substrates for calcium-phopholipid-dependent kinase suggests that the effects might be modulated by phosphorylation and dephosphorylation (Pyerin et al., 1987).

Other phase II drug metabolizing enzymes for which consistent increase in GSF and MCF has been described are uridine-diphosphate-gluronyl-transferase (Bock et al., 1982; Fischer et al., 1983) and microsomal epoxide hydrolase (Enomoto et al., 1981; Levin et al., 1978; Buchmann et al., 1985; Tsuda et al., 1987). This latter is linked to hypomethylation of the gene (Ding et al., 1990). Epoxide hydrolase may be importance in toxicity because of effects on toxic bile acid transport, mediating for example transport of taurocholate into hepatocyte smooth endoplasmic reticulum vesicles (Alves et al., 1993). Expression of epoxide hydrolase isoenzymes in APF has yet to be ascertained but in the context of hormonal control it should be mentioned that diabetes and starvation bring about an increase in the cytosolic form while decreasing the microsomal form, along with glutathione S-transferases (Thomas et al., 1989).

Resistance of prenoplastic cells could also be relaated to the reported increase of DNA repair in liver nodules (Citti et al., 1990) with enhanced activity of 06-methylguanine DNA alkyltransferase (Becker and Planche-Martel, 1986). However, this was not supported by the findings of Ozaki et al (1993) in terms of the rapidity with which adducts are removed.

e) Signal transduction

The loss or decrease of adenylate cyclase activity reported in nitrosomorpholine-induced lesions (Ehemann et al., 1986) suggests a shift in regulatory mechanisms away from cAMP-dependent pathways and PKA. However, in Solt-Farber lesions the enzyme appears to be increased (De Canniere et al., 1992). The finding of increased PKCa/b (La Porta et al., 1993; 1994) but not PKCd (La Porta et al., 1995) on the other hand is indicative that the phospholipid pathway is switched on.

Expression of the insulin receptor substrate 1 (IRS-1) has been found to be increased in GSF but not APF (Nehrbass

et al., 1998;1999).Insulin-like growth factor II becomes re-expressed during hepatocarcinogenesis induced by 3MeDAB (Ueno et al., 1988) and also in woodchuck nodules (Yang and Rogler, 1991; Yang et al., 1993) and in precancerous lesions in transgenic mice with antithrombin III-SV40 gene (Cariani et al., 1991).

The involvement of alterations in oncogenes in hepatocarcinogenesis in rodents and man has been reviewed (Mehta, 1995). One study demonstrated increase in foci and nodules (Galand et al., 1988), and another in nodules (Beer et al., 1986), but not in Solt-Farber lesions despite increases in c-myc and c-fos (Posch-Hallstrom et al., 1989). Thus, the presence or absence of farnesyl PP may be a more important determinant of signalling than the absolute level of c-ras expression (Maltese, 1990).

The possibility that the central signalling pathway is indeed switched on, however, is indicated by the elevated c-raf expression found in FAH induced by NNM (Strobel et al., 1990) and other carcinogens (Beer et al., 1988; Huber et al., 1989). Increase has also been documented for c-erb (Alexandre et al., 1990).

The increment in density of nuclear pores in foci (Sugie et al., 1994) might also reflect increased trafficking between the nucleus and the cytoplasm. Within the nucleus, a common finding is elevated expression of c-myc in rat foci and nodules induced by the Solt-Farber system (Nagy et al., 1988; Huber et al., 1989; Porsch-Hallstrom et al., 1991). N-myc 2 is coordinately expressed with insulin-like growth factor II on precancerous altered hepatic foci in woodchuck hepatitis virus carriers (Yang et al., 1993). Elevated c-fos has also been described in hepatic nodules (Corral et al., 1985; Simile et al., 1994) and c-jun in approximately one third of focal lesions (Suzuki et al., 1995)

Focus Physiology and Processes Underlying Hepatocarcinogenesis

The two major phenotypes of foci induced by carcinogenic regimens in the rat liver demonstrate separate directed and apparently co-ordinated shifts in biochemistry.Many of the changes in enzyme expression observed in preneoplastic hepatocyte foci are reminiscent of the influence of insulin in the liver. Thus, catabolic processes appear to be downregulated while anabolism is favoured (see Fig 4). Indeed, a direct role for insulin in inducing such lesions is indicated by the hepatomegaly and massive glycogen storage associated with high dose hormone treatment (Nakamuta et al., 1993) as well as the experimental findings with islet implants, which eventually cause tumors (Dombrowski et al., 1994; 1997). Interestingly, implants of thyroid or ovarian tissue also produce hyperproliferative lesions, this time reminiscent of APF (Dombrowski et al., 2000; Klotz et al., 2000).

Recently, attention has been drawn to the possible role of insulin as a promoter of neoplasia (Bruning et al., 1992; McKeown-Eyssen, 1994; Giovannucci, 1995; Kazer, 1995, Moore et al., 1998). The question of whether the biochemical phenotype of preneoplastic foci in the liver may be in some way linked to altered hormone sensitivity has, however, received only scant regard up till the present (Bannasch et al, 1997; Pearline et al., 1996).

In addition to the direct proof of stimulation of hepatocarcinogenesis provided by Dombrowski in a series of papers, there are a number of lines of evidence in favour of insulin being a promoter of neoplasia in the liver. In terms of focal lesion and tumor development associated with hormonal imbalance in man, three disease states clearly deserve mention.

Firstly, the association between type I (von Gierke's) glycogen storage disease and liver cancer development is of obvious significance (Bannasch et al., 1997; Bianchi, 1993). In this case the insufficient glucose-6-phosphatase activity leads to glycogenosis, and glycogen-rich lesions with a background of elevated glucagon in the blood (Greene, 1982; Moses; 1995). The second disease is leprechraunism (Donohue's syndrome), due to mutations in the insulin receptor gene (Elsas, 1985), characterized in some cases by multiple small nodules in the liver, composed of large pale hepatocytes with a great quantity of glycogen and a little fat (Ordway and Stout, 1973).

The third disease is cirrhosis, whereby in man a persistent increase in serum insulin levels has been reported, caused by reduced degradation along with higher secretion (Letiexhe et al., 1993). Also in rats, CCl4-induced cirrhosis is associated with lower serum glucose and elevated insulin, in this case largely due to reduced clearance (Wu et al., 1994). Dietary restriction and reduced body growth also correlate with decrease in foci and tumor development as well as insulinaemia (Lagopoulos et al., 1991).

With the long periods required before neoplasms become detectable, any relative advantage that the preneoplastic phenotype gives to the constitutent cells, whether it be because of increase in the inherent ability to progress through the cell cycle, an enhanced responsiveness to stimulatory factors, or a decreased relative propensity to undergo apoptosis, could be of interest to preventive efforts. Regarding chemoprevention or alteration of the lifestyle, the findings for preneoplastic lesions in the liver would thus suggest that measures taken to reduce hyperinsulinemia might be effective, including both increased intake of soluble fibre in the diet and frequent exercise (Moore et al., 1998a-c). In addition, insulin sensitizers and compounds targeting particular physiological pathways, like for example HMG-CoA reductase inhibitors, might deserve especial consideration.

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