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Advances in liver pathology

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Liver biopsy in 2000. The pathologist's view

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In spite of tremendous progress in virology and molecular biology with numerous applications in noninvasive diagnostic methods, at the end of this millennium liver biopsy still remains the gold standard by which other modalities to evaluate the liver are judged (1).

In the year 2000 – as before – the pathologist has to remain aware that the diagnostic usefulness of liver biopsy changes over time (2). He or she may expect to be less often confronted with pathology of acute hepatitis, and problems of differentiation between intra- and extrahepatic cholestasis, and other conditions such as hemangiomas and focal nodular hyperplasia; these diagnostic problems will be resolved by the clinician with other methodologies such as serology and imaging techniques. Instead, he or she will have to acquire more expertise in problems inherent to liver transplantation and to acquired immunodeficiency (2).

The diagnostic accuracy of liver biopsy can be markedly improved when appropriate care is taken to minimize pitfalls in specimen handling and to maximize interpreter performance (3). For these goals, as in the past, an intelligent collaboration between clinician and pathologist is imperative. As such medical teamwork is still rather the exception than the rule, further integration of pathology into clinical medicine will remain a necessity in the next millennium.

Besides the classical indications and methods of examining and reporting liver biopsies, the pathologist active at the turn of the millennium should pay particular attention to actual and future needs. The following lines mention a nonexhaustive enumeration of some general examples.

Liver biopsy will remain useful in chronic liver diseases, as noninvasive methods for assessment of liver fibrosis remain inadequate (4). However, for the pathologist this implies that grading of disease activity and staging of disease progression should be specified as much as possible, and not only in chronic hepatitis (5). In the appropriate setting, grading and staging can be achieved with the use of semiquantitative scoring systems (6). As the number of new drugs of potential use in liver disease will undoubtedly increase, there will be a growing need for further refinement of semiquantitative scoring procedures for comparison of pre- and post-treatment states. With further progress in computer-assisted analysis and digital display, automated and interactive image analysis will become of greater diagnostic importance. Presumed first applications are quantitation of liver fibrosis and pattern recognition diagnosis of liver cirrhosis.

Refinements of biopsy techniques will render the biopsy procedure less dangerous, also in view of tumor spread in case of liver malignancy (7). As a result, the pathologist will have to deal more and more with early and premalignant changes in liver tumor pathology; this will in parallel increase his/her role in patient care and surveillance. An example is the investigation of "irregular regeneration" in chronic viral hepatitis 0 (8). The diagnostic yield of liver biopsy will further be increased by application of immunohistochemistry and *in situ* hybridization. Further work is needed to increase the number of new applications and to improve the reliability of existing ones *(e.g., in situ* demonstration of viral antigens in viral hepatitis C, and search for markers of autoimmune hepatitis).

Introduction of immunostains in various diagnostic areas leads to better yields in finer diagnostic precision. Examples are immunostains for "bile duct-type" cytokeratins in chronic cholestatic liver disease, and demonstration of proteins in endoplasmic reticulum storage disorders (9).

Thoughtful application of immunostaining and electron microscopy may assure original results, like recognition of hitherto unrecognized tumor entities (10).

As was the case before, one may guarantee that liver biopsy will reveal an unexpected diagnosis (11), or unveil additional pathology superimposed on the main clinical suspicion (3).

Above all, pathologists should continue to use their inventiveness in order to meet the challenge of the future and to maintain the position of liver histopathology as the gold standard and cornerstone in the diagnosis of liver disease.

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Natural history of liver fibrosis

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Liver fibrosis is the hallmark of all chronic liver diseases; failure to eliminate a deleterious agent (virus, toxic, autoantigen) leads to chronic inflammation with liver fibrosis and cirrhosis as an end point. Pathogenesis of cirrhosis is therefore close to other destructive fibrosis, such as chronic gastric ulcer, hypertrophic scar or pulmonary interstitial fibrosis.

The liver extracellular matrix

Liver fibrosis is the result of the accumulation of extracellular matrix components (ECM) with subsequent destruction of liver architecture and liver cell dysfunction. ECM is a complex network composed of three main groups of macromolecules: the collagens, the adhesive glycoproteins and the proteoglycans (1). Among the most abundant proteins are those of the collagen family (mainly type I, III, IV, V, VI and XVIII in the liver). From a histopathological point of view, these different collagen isotypes form either interstitial or fibrillar collagens (collagen type I, III and V) or basement membranes (type IV collagen). Among the glycoprotein family, most important are laminin (an other major constituent of basement membranes) and fibronectin. These molecules serve not only as a scaffold for epithelial liver cells but also have specific functions according to their modular architectures. Fibronectin is involved in cell attachment, differentiation and migration through its different domains, each involved in interaction with specific ECM component. Proteoglycans, the third major group of ECM molecules are composed of a protein backbone linked to sulfated polydisaccharides (glycosaminoglycans). They are located in the interstitium or in cell membrane, either transmembranous or pericellular. Liver ECM is also rich in cytokines, growth factors, or serum macromolecules that are sequestrated into ECM through specific interactions with various components, mainly proteoglycans and adhesive glycoproteins. Transforming growth factor-ß (TGF-ß), a major growth factor involved in fibrogenesis is bound to ECM in an inactive form. Under certain circumstances, these molecules can be activated and serve as a local reservoir of active molecules.

Hepatic stellate cells (HSC) are the major cellular source of EOM molecules (2). They are located in the Disse's space. In the normal liver, the function of HSO is to store vitamin A. Under various stimuli (disruption of cell-cell or cell-matrix contacts, growth factors such as TGF-ß, reactive oxygen species, lipid peroxidation products, acetaldehyde), these cell undergo phenotypical transformation to transitional cells with a myofibroblast phenotype. In this condition, HSC produce a large amount of the different EOM components. Endothelial cells but also fibroblasts of the portal tract are also involved in ECM production.

It is now feasible to isolate and grow HSC *in vitro.* This approach makes it possible to study in detail the multiple molecules involved in HSC activation as well as their signal transduction pathways. With this model, it has been showed that TGF-ß is the major fibrogenic molecule inducing transcription of most of the EOM molecule-associated genes (collagen, fibronectin, laminin) and that platelet-derived growth factor (PDGF) is the major mitogenic molecule for HSC. Other cytokines and growth factors such as fibroblast growth factor-2 (FGF-2), interleukin-10 (IL-10) and interferon-gamma are also involved in the regulation of these genes as well as in HSC proliferation. These studies have also allowed for the development arid the study of antifibrogenic agents which have also been tested on different experimental models of liver fibrosis. Antioxidants, antisense or soluble receptors of growth factors are being tested, some of them with promising results.

How to evaluate liver fibrosis

Liver biopsy is the gold standard for assessing liver fibrosis. However, sample variability of fibrosis is a major drawback in fibrosis assessment. Liver fibrosis is a diffuse lesion, it is closely associated with liver regeneration so that areas of dense fibrosis may coexist with areas of liver cell regeneration leading to irregular distribution of fibrosis. Such variability in the distribution of fibrosis precludes the use of overly precise objective methods of fibrosis measurement on liver core biopsy (quantitative image analysis). The use of less accurate but more reproducible scoring systems, such as those proposed in the different scoring systems of chronic hepatitis, are much more adapted to assess fibrosis with confidence in clinical practice. Theoretically, the use of serum markers of liver fibrosis would allow a global assessment of the fibrogenesis process. Unfortunately there is no marker of fibrosis stage or fibrogenesis with sufficient sensitivity and specificity to have high positive or negative predictive values.

Can fibrosis regress

Strong biochemical arguments suggest that the liver contains enzymatic equipment allowing the destruction of ECM. Matrix metalloproteinases (MMP) are a family of proenzymes that can destroy the different ECM components (3). Several studies have shown overexpression of MMP in various conditions associated with liver fibrosis. MMP are produced by HSC, the same cell type involved in EOM production. These cells also produce tissue inhibitors of metalloproteases (TIMP), a set of molecules that inhibit MMP activity. Therefore, evaluation of matrix degradation remains a difficult task.

There are, however, strong experimental and clinical arguments showing that liver fibrosis can regress. In the 0014-induced liver cirrhosis in rat, the arrest of 0014 administration allows a near complete fibrosis regression. At this point, it must be remembered that cirrhosis is composed of the association of both annular fibrosis and liver cell regeneration. It is also evident, from human and experimental studies, that liver regeneration begins very early after liver insult, well before the cirrhotic stage. There is now strong evidence showing that liver fibrosis regression can occur both from ECM degradation and liver regeneration. It can be hypothesized that at a stage of stable fibrosis (in the absence of ongoing fibrogenesis), liver cell regeneration can compress and disrupt fibrous septa leading to a restitution of near normal liver architecture with portal tract and centrilobular veins. As a matter of fact, a recent experimental study showed that gene therapy using a hyperglycemic-glycogenolytic factor (HGF) retroviral vector induced the complete disappearance of the cirrhotic architecture in an animal model of fibrosis.

Natural history of liver fibrosis
Chronic hepatitis C as a paradigm

Chronic hepatitis C virus infection (HCV) is estimated to affect 170 million individuals worldwide. Thirty percent of these individuals will eventually develop cirrhosis. HCV can be lethal almost exclusively when it leads to cirrhosis. Therefore, an estimate of fibrosis pro-

gression represents an important surrogate end point for evaluation of the vulnerability of a given patient and for assessment of treatment impact on natural history. Activity grade is not very useful to predict fibrosis progression since there is no study demonstrating that activity is predictive of fibrosis progression independent of fibrosis stage and, in fact, fibrosis alone is the best marker of ongoing fibrogenesis (4). Because of the informative value of fibrosis stage it is worthwhile for clinicians to assess the speed of the fibrosis progression. In a large population we observed that fibrosis progression rate was not normally distributed with an asymmetrical distribution suggesting the presence of at least three populations: one population of "rapid fibrosers", a population of "intermediate fibrosers" and one population of "slow fibrosers" (5). Using the- median fibrosis progression rate, and without treatment, the median expected time to cirrhosis was 30 years; 33% of patients had an expected median time to cirrhosis of less than 20 years and 31% will never progress to cirrhosis, or would do so in more than 50 years. There are no clear cut explanation to the individual susceptibility of developing liver fibrosis. However several factors have been clearly shown as associated with higher fibrosis progression rate: duration of infection, late age, male gender, consumption of alcohol and HIV coinfection with low CD4 count. For example, the estimated probability of progression per year for men aged 61-70 years was 300 times greater than that for men aged between 21 and 40 years and 10 times greater than that for women aged 61-70 years. Virus-associated factors such as genotype, viral load, quasi species are not associated with fibrosis.

Better knowledge of factors associated with individual susceptibility will allow a better definition of the patients' risk of developing fibrosis.

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Alcoholic and nonalcoholic steatohepatitis

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Chronic alcohol abuse may lead to two different types of liver injury. Most drinkers develop fatty liver, which by itself is a reversible alteration and has a low risk of progressing to liver cirrhosis. Approximately 20-40% of heavy drinkers, however, develop a special type of alcoholic liver disease, namely alcoholic steatohepatitis (ASH), which rapidly progresses to liver cirrhosis in most of the affected patients. ASH is characterized by the ballooning of hepatocytes, steatosis, hepatocellular necrosis and apoptosis, pericellular and perivenular fibrosis, inflammation with predominantly polymorphonuclear granulocytes, cholestasis and activation of Kupffer cells (1). Furthermore, a hallmark lesion seen in hepatocytes is the appearance of cytokeratin (CK)-containing cytoplasmic inclusions, termed Mallory bodies (MBs), which is accompanied by a disruption of the CK intermediate filament cytoskeleton (2-4). These alterations, however, are not specific for ethanol-induced toxic liver injury, but can also be found in patients without evidence of alcohol abuse. Occurrence of nonalcoholic steatohepatitis (NASH) is associated with obesity, noninsulin-dependent diabetes, intestinal bypass surgery, bacterial contamination of the small bowel, as well as with several drugs, such as amiodarone or perhexiline maleate, which are known to be inhibitors of mitochondrial ß-oxidation (5). Identical hepatocytic alterations to that seen in human liver biopsies with ASH or NASH can be experimentally induced in mice by chronic intoxication with the porphyrogenic drugs griseofulvin (6) or 3,5-diethoxcarbonyl-1 ,4-dihydrocollidine (DOG) (7). Analysis of the protein composition of MBs revealed that besides CKs also non-CK components, namely, the stress-inducible MM 120-1 antigen, a 62-65 kDa MB component recognized by the antibody SMI 31, and ubiquitin, which is a common constituent of a variety of cytoplasmic inclusions occurring in different chronic degenerative diseases, are present in MBs (8, 9). The role of these different components in MB formation as well as the relevance of MBs and the cytoskeletal alterations in the course of alcoholic hepatitis is still unclear.

To obtain further insight into the role of the different MB components and the alterations of the OK cytoskeleton in the pathogenesis of ASH and NASH, we investigated mice in which either of the two OK genes expressed in hepatocytes, namely CK8 and CK18, had been inactivated (10, 11). Since OK intermediate filaments are obligatory heteropolymers, no intermediate filaments can be formed in the absence of one of these two partner proteins resulting in hepatocytes devoid of a cytoplasmic OK network (12, 13). DDC intoxication of *CK8—/—* mice showed a higher toxicity than in wild-type mice. After 3 months of intoxication seven out of 12 CK8—/— animals had died, whereas all 13 K8+/+ mice survived. Analysis of metabolic alterations in these mice revealed that mice lacking CK8 develop a much severer porphyria than wild-type mice. Analysis of livers from DDC-fed CK8-/— mice with double-label immunofluorescence microscopy showed that in the absence of CK8 no MBs were formed. Moreover, none of the non-OK MB components accumulated in these livers, indicating that OK is the core protein in MBs and that all other MB components either bind to or coassemble with CK. These *in vivo* data are in line with previously obtained *in vitro* data where we found that overexpression of CK by transient transfection of cells is sufficient to lead to induction of the MM 120-1 protein and to association of the MM 120-1 protein with cytokeratin aggregates, mimicking the initial phase of MB formation. To our surprise, DDC intoxication of mice with only one inactivated CK8 allele did not lead to alterations of the cytokeratin cytoskeleton nor to the appearance of MBs, although all other signs of DDC intoxication such as the loss of lamin B2 from the nuclear lamina, development of porphyria, proliferation of bile ductules were present. This different behavior of wild-type and heterozygous CK8 mutant mice has, therefore, to be attributed to the loss of one CK8 allele. To obtain more information on the functional consequences of the disruption of one CK8 allele, the mRNA concentrations of CK8 and OKi 8 were analyzed using a quantitative RT-PCR assay. DDC intoxication led to an approximately 5-fold overexpression of both CK8 and CK18 mRNAs in wild-type mice. In CK8+/— mice, DDC caused a similar increase in the concentration of 0K18

mRNA. The concentration of 0K8 mRNA, however, raised only to approximately 50% of OKi 8 mRNA level. This indicates that in the absence of the second CK8 allele the DOG-induced overexpression is half maximal, and that in the situation of a relative excess of 0K18 over 0K8 no MBs are formed. Furthermore, these data showed that the effect of DOG intoxication on CK mRNA expression is independent from the GK cytoskeleton, suggesting that the overexpression reflects a direct or indirect response to the toxic injury.

Further important clues as to the role of GK in alcoholic liver disease have come from experiments in GK18 knockout mice. CK18—/— mice responded completely differently to DOG intoxication than did GK8 knockout mice. Although CK18-/— like GK8—/— mice are devoid of a CK IF cytoskeleton, CK18-/— animals showed no increased sensitivity to DOG. Therefore, the lethality seen in DOG-treated CK8-deficient mice cannot be attributed to the loss of the CK cytoskeleton but has to be related to the imbalance of nonassembled GK polypeptides or CK oligomers. This suggests a completely new mechanism for how CK may interfere with cellular processes. The specificity of these interactions is underlined by the fact that there are substantial differences in the consequences for the liver between mice which lack GK8 and mice which lack GK18. These differences are also pertinent with respect to MB pathogenesis. In contrast to GK8 defective mice, DOG intoxication of CK18-/— mice led to the formation of classical MBs consisting of CK8, the MM 120-1 and SMI 31 protein, and ubiquitin. These findings further show that CK8 can be stabilized in cells under certain pathological conditions even without the corresponding partner CK.

In conclusion, from these experiments it is obvious that CK8 is the key protein in MB formation. The increased toxicity in DOG-fed heterozygous and homozygous GK8 gene-deleted mice widens our view of the cellular function of GK and demonstrates that overexpression of GK8 protects hepatocytes from toxicity. Moreover, the fact that mice which were able to form MBs had fewer signs of toxicity than those which did not form MBs implies that the MB itself is not detrimental to the hepatocyte but rather is a product of a new cellular defense mechanism involving GKs.

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Hemochromatosis: Recent molecular findings

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Hemochromatosis is the clinicopathological syndrome caused by toxic accumulation of iron in the tissues: it may be demonstrably hereditary, sporadic or associated with another disorder, "secondary". Hemochromatosis causes liver disease, endocrine failure, joint disease and cardiomyopathy and is a true multisystem disease. Secondary hemochromatosis occurs when excess iron is administered or taken up by the intestine as a little understood effect of chronic dyserythropoietic anemia. Early treatment of iron overload prevents irreversible liver damage and is associated with normal life expectancy, provided cirrhosis has not supervened.

Hereditary hemochromatosis (HFE) is characterized by onset in the adult, juvenile (<35 years) or neonatal periods. Recently, the class I human lymphocyte antigen (HLA)-linked HFE gene, initially termed HLA-H, for the prevalent adult form of hemochromatosis that maps to chromosome 6p21. .3 has been identified by Feder *et al.*. This gene encodes a class I gene product that is almost ubiquitously expressed on cell membranes and specifically on the basolateral membrane of intestine epithelial cells. Two mutations in HFE, Cys282->Tyr and His83->Asp, have been associated with adult hemochromatosis, although the principal mutation Cys282->Tyr (C282Y) disrupts a ß 2-microglobulin binding domain essential for cell surface expression of class I molecules: this is the major determinant of iron overload. Between 85% and 10-0% of adult hemochromatosis patients are homozygous for C282Y and as predicted, the carrier frequency is —5% of the population. Studies in several pedigrees however have failed to find G282Y in some adult and most juvenile patients with hemochromatosis. Moreover, some adult C282Y homozygotes may have no signs of disease or abnormal parameters of iron metabolism. H63K appears to be a minor determinant of disease. Penetrance of the C282Y mutation in homozygous form, increases with age and about two-thirds of men over the age of 40- appear to show signs of iron-related tissue injury and/or organ failure. Pedigree analysis has also shown that some juvenile patients with severe iron overload have a disease gene with no linkage to chromosome 6p markers.

The identification of C282Y in HFE undoubtedly represents an important step in understanding iron metabolism, although beyond our knowledge that ß2-microglobulin knock-out mice develop iron storage disease and liver tumors and pathology that resembles the human disease, the role of HFE is quite unknown. Clearly the G282Y mutation is neither sufficient to explain all instances of human iron

overload and has variable expressivity. Thus, operational tests of iron status [% saturation of transferrin (—90% of patients); serum ferritin (—70% of patients)] remain useful for diagnostic screening. Liver biopsy and tIssue iron quantification remain for the present the gold standard tests for all forms of hemochromatosis. For our understanding of iron metabolism, the identification of HFE represents a promising step but the means by which loss of HFE expression at the cell surface leads to systemic iron storage remains elusive. The identification ofthe mammalian intestinal iron uptake and red cell iron transport protein, NRamp 2, provides a tantalizing potential link for a molecular understanding of the control ot body iron balance. Lately, the identification of hephaestin, a ceruloplasmin-like protein involved in the efflux of iron from intestinal epithelial cells, may add much more to our portfolio of contributors to iron physiology; hephaestin is mutated in the sex-linked anemia mouse that is defective in iron transport from intestine to plasma.

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Stem cells in liver carcinogenesis

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Neoplastic development in the liver

Genesis of liver tumors most probably occurs via multiple molecular mechanisms which depend on both the nature of the carcinogen and the lesions induced by it. The liver system can be viewed as being Composed of two stem Cell systems: the unipotential (possibly bipotential) hepatocytic and the multipotential nonparenchymal epithelial (ductular) systems (1, 2). Therefore, it seems reasonable to expect that both cell systems could provide progenitor cells for the neoplastic process. There is no doubt that the hepatocyte frequently is the progenitor cell for liver tumors (3). The involvement of the nonparenchymal (ductular) system in the genesis of liver tumors, particularly hepatocellular carcinomas, is still hotly debated. On the one hand, it has been proposed that "the cell of origin of liver cancer is the putative liver stem cell or its progeny, the transitional duct cell" (4). Alternatively, Farber (3) has stated that "rare original mature hepatocytes in zone 1,2 or 3 of the adult liver appearing after initiation with genotoxic carcinogens have been shown to be the cell of origin for foci or islands of altered hepatocytes and of nodules derived from these foci." The central issue in better understanding the involvement of the nonparenchymal epithelial (ductular) cells in the carcinogenic process is the characterization of the mechanisms that regulate both the proliferation of these cells after carcinogenic as well as noncarcinogenic insults, and the factors governing the lineage commitment processes in this compartment.

Hepatic stem cells and hepatocarcinogenesis

A landmark contribution to the involvement of nonparenchymal epithelial cells in hepatocarcinogenesis was provided by Farber (5) who provided a detailed description of the early histological changes during hepatocarcinogenesis caused by three chemical carcinogens. The carcinogens used by Farber, ethionine, 2-acetylaminofluorene (AAF), and 3'-methyl-4-dimethylaminoazobenzene (Me-DAB), in spite of being structurally very different, caused similar histological alterations. The common features included: i) oval cell proliferation which progressively involved most of the liver lobule, beginning in the portal areas; ii) degenerative and hypertrophic changes in hepatocytes adjacent to proliferating oval cells, and iii) nodular regenerative hyperplasia of liver cells. There were, however, important differences in the time course of appearance and fare of the oval cells induced by these three hepatocarcinogens. While oval cells appeared early following ethionine and AAF administration (7 and 14 days, respectively), their appearance occurred significantly late after Me-DAB treatment (first seen at day 21). More importantly, the fate of the oval cells in the Me-DAB-treated animals were different from those induced by ethionine and AAF, In the early stages the oval cells induced by Me-DAB were morphologically indistinguishable from those generated by ethionine and AAF. However, at later stages areas of apparent transition between oval cells and hepatocytes were numerous in the Me-DAB treated animals but absent in those receiving ethionine and AAF.

These observations raise several important issues. First, and most importantly, it is now well established that many different Chemical compounds capable of producing liver tumors in rats and mice, induce a similar sequence of histological changes in which oval cell hyperplasia is prominent (6). Secondly, if the transition from oval cells to hepatocytes can be morphologically observed after Me-DAB treatment, then it is in principle established that oval cells (or at least a subpopulation of oval cells) have the capacity to differentiate into hepatocytes. The fact that administration of ethionine or AAF did not provide the same clear morphological sequence as seen with Me-DAB in which the oval cells merge imperceptibly and were in continuity with the regenerating nodules, suggests that the compounds capable of inducing oval cell proliferation may greatly affect both the rate and extent of oval cell differentiation into hepatocytes. The tact that a large population of oval

cells is cycling during the early stages of chemical hepatocarcinogenesis and that these cells can differentiate into hepatocytes strongly suggests that at least a percentage of the hepatocellular carcinomas are derived from oval cell progenitors. Recently, there has been accumulating experimental evidence in support of this notion. Hixson et al. (7, 8) have used a battery of monoclonal antibodies specific for antigens associated with bile duct cells, oval cells and fetal, adult, and neoplastic hepatocytes to analyze the phenotypic relationship between oval cells, foci, nodules, and hepatocellular carcinomas during chemical hepatocarcinogenesis. These investigators found, using the resistant hepatocyte model of Soft and Farber (9), that oval cells, gamma-glutamyltransferase-positive hepatocellular foci, persistent hepatocyte nodules, and primary hepatocellular carcinomas express both oval cell and hepatocyte antigens. This finding indicates a precursor product relationsship between oval cells and carcinomas. Similar results were obtained by Dunsford *et al.* (10) using different monoclonal antibodies raised against oval cells. These lineage relationships between oval cells and hepatocellular carcinomas also exist in other models of liver carcinogenesis. For example, animals maintained on a ODE diet display markers for oval cells and hepatocytes in a significant percentage of nodules and hepatocellular carcinomas (7, 8). Also, metastatic foci in the lung from the animals harboring these liver tumors show essentially the same phenotype (7).

The evidence for oval or ductular cells as progenitors for hepatocellular carcinomas- is not restricted to experimental models of chemical hepatocarcinogenesis in rodents. Results from Van Eyken et *al.* (11) on the cytokeratin expression in 34 "classical" human hepatocellular carcinomas (HOC) using monospecific anticytokeratin antibodies show that all HOGs were positive for cytokeratins 8 and 18. However, in 17 cases a variable number of the tumor cells were positive for cytokeratin 7 (two cases), cytokeratin 19 (7 cases), or both 7 and 19(8 cases). The authors also reported that only three of 11 well-differentiated tumors display an "unexpected" pattern of immunoreactivity as opposed to seven of seven poorly differentiated tumors. This is particularly important in light of the earlier observation by Denk et *al.* (12) that cytokeratins continue to be expressed when hepatocytes become neoplastic. These observations are also highly relevant in light of the recent findings of Hsia *et al.* (13) and Vandersteenhoven *et al.* (14) who demonstrated immunohistochemically the presence of ductular "oval" type cells with characteristics of both bile ducts and hepatocytes in the liver of patients with end stage cirrhosis and/or tumors from hepatitis B infection. It is important at this stage to reemphasize that the relative percentage of primary hepatocellular carcinomas derived from oval cell progenitors vary over a wide range depending on the protocol of the carcinogen and/or the chemical carcinogen used as well as the extent of oval cell involvement in the early stages of the process.

Transformation of liver derived epithelial (oval) cells

The most direct evidence- that oval cells and/or rat lung epithelial (RLE) cells can progress to hepatocellular carcinomas comes from *in vitro* transformation of these cells. Spontaneous transformation of RLE and oval cells as well as transformation with chemical carcinogens and dominant oncogenes results in the tumors displaying a wide range of phenotypes including well-differentiated hepatocellular carcinomas, cholangiomas, hepatoblastomas, and poorly differentiated or anaplastic tumors (15-18). In one of the most comprehensive studies on the chemical transformation of the RLE cells by Tsao and Grisham (15), a wide range of tumors described including carcinomas, sarcomas, mixed epithelial-mesenchymal tumors, and undifferentiated tumors. In addition, several tumors were morphologically indistinguishable from hepatocellular carcinomas.

We have recently demonstrated that cytokeratin 14 is expressed in several RLE cell lines (19). Although the partner for cytokeratins 8 and 14 has traditionally been found to be cytokeratins 18 and 5, respectively, it is now well documented that cytokeratins 8 and 14 can be expressed in the complete absence of their traditional partner (20-22). We have shown that in some RLE cell lines cytokeratins 8 and 14 form heterotypic filaments (21). We also found that these cell lines express vimentin along with the cytokeratins (22). However, the spontaneous transformation and differentiation of one of our RLE cell lines to a hepatoblast-like phenotype, forming a well-differentiated trabecular hepatocellular carcinoma, results in an abrogation of vimentin protein expression and a change in cytokeratin expression from which cytokeratin 14 was substituted by 18 (23). We have used this RLE transformation system to study the relationship between the expression of cytokeratins 14, 8, as well as 18 and alpha-fetoprotein (AFP) during the process of proliferation and differentiation of the RLE cell line to a hepatoblast-like progeny.

The steady-state levels of mRNA transcripts for cytokeratin 14 and AEP, as well as for cytokeratins 8 and 18 and vimentin show a significant change in the expression pattern during the process of transformation (23). Before the cells display morphological signs of transformation, a high steady-state level of cytokeratin 14 transcripts in addition to transcripts for cytokeratin 8 and vimentin is detected. During the process of transformation that occurred within 33-35 passages, the steady-state levels of cytokeratin 14 and vimentin abruptly declined, and could not be detected in later passages nor in the clonal transformed B5T cell line. The disappearance of the cytokeratin 14 and vimentin mRNA transcripts closely corresponds with the appearance of a 2.1 kb transcript for AFP and a 1.4 kb transcript for cytokeratin 18. The mRNA transcripts for cytokeratins 8 and 18 as well as those for AFP are present in the transformed clonal B5T cell line. In contrast to the spontaneous transformation, these same RLE cells when transformed by dominant oncogenes yield primitive and anaplastic tumors (16). These data indicate that the tumor phenotypes derived from RLE and/or oval cells may depend on both the mechanism of transformation and the stage of differentiation of the cells when the transformation occurs.

Conclusions

The adult organism contains many kinds of stem cells that exist at different stages of differentiation and have very different capacities for generating multilineage progeny. The capacity for self-maintenance is a fundamental and common trait of all stem cells. A cell population that has an extensive self-maintaining capacity is the only definition that applies to all stem cells. It is proposed that the liver system be viewed as composed of two stem cell systems: the unipotential hepatocytic and the multipotential nonparenchymal epithelial (ductular) systems. Although the participation of the nonparenchymal epithelial system in the development of liver tumors is still not fully defined, strong evidence now exists indicating that both these systems can and do provide progenitor cells for the neoplastic process in the liver. The central issue in better understanding the involvement of the nonparenchymal epithelial (ductular) cells in the carcinogenic process is the characterization of the mechanisms that regulate both the proliferation of these cells after carcinogenic as well as noncarcinogenic insults and the factors that govern the lineage commitment processes in this stem cell system.

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Hepatocellular carcinoma

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Hepatocellular carcinoma (HOC) is the most common primary tumor of the liver worldwide. Its incidence is in the region of 1,000,000 new cases per year, most of which occur in Southeast Asia and tropical Africa. Mortality is nearly 100%, except for a small number of early cases detected by screening. There is a heavy male preponderance in all areas.

Etiology and pathogenesis

Hepatic carcinogenesis has been extensively investigated in experimental animals, mainly by the use of chemicals. These studies established the multistep nature of malignant transformation through initiation, promotion and progression. The findings are now increasingly applied to humans.

The list of etiologies is quite long (Table 1) but chronic hepatitis B (HBV) and hepatitis C (HCV) infections respectively account for 70-75% and 10-15% worldwide. HBV remains predominant in Asia and Africa but the proportion of HCV-related cases is rising, particularly in Japan and the Middle East. HBV transmission occurs early in life and HOC develops in young middle age; HCV is acquired in adulthood and patients are 15-20 years older. Aflatoxin is an important factor or cofactor in certain areas such as Mozambique and the Qidong province of China. Alcoholic cirrhosis accounts for 50-70% of cases in low-incidence Western countries alone or in combination with HBV or HCV. Inherited metabolic diseases are rare but some, *e.g.,* tyrosinemia, hemochromatosis, carry a surprisingly high risk, Indeed, liver cancer is the only malignancy that complicates these disorders regularly. Diabetes mellitus, a polygenic condition, has recently been added to the list.

Tumor cells of HOC show evidence of severe genomic instability by chromosome breakages, duplications and translocations. Molecular biological studies of the role of HBV have been the most interesting, if not yet conclusive. While this role may be indirect, as 70-90% of HBV-related HOC develop through cirrhosis, HBV may act directly. It is a DNA virus that integrates in liver cell nuclei in a random fashion but at sites that are close to growth controlling genes and may result in their dysfunction. It was first thought that this mechanism, resembling insertional mutagenesis of retroviruses,

Table 1. Etiology of hepatocellular carcinoma.

Hepatitis B virus (HBV)
Hepatitis C virus (HCV)
Aflatoxin
Alcohol
Inborn errors of metabolism
Tyrosinemia
Glycogen storage disease
Porphyria
alpha-1-antitrypsin deficiency
Hemochromatosis
Synthetic gonadal steroids
Membraneous obstruction of the inferior vena cave
Radiation

was largely responsible but no consistent pattern ever emerged. More recently, two trans-activating proteins have been found, one being the product of the pre-S2 region of the HBV S gene and the other of the HBV X gene. Of the two, the latter is of the greater interest. Most HOC and cell lines derived from them contain transcripts of the X gene either as activated oncogenes of the *ras, myc* and *fos* families, or as inactivated tumor suppressor genes notably p53 and possibly Rb. The X gene may also have many other effects on growth promoters, protein kinases and DNA repair. Indeed, its activities have been described as "promiscuous. HBV-like viruses in animals that do not possess the X gene do not give rise to HOC; on the other hand, X gene transactivators lead to liver cell tumors in transgenic mice.

HCV is an RNA virus that cannot integrate into the DNA of liver cells and its role in hepatic carcinogenesis is likely to be that of a chronic necroinflammatory agent causing cirrhosis. "Irregular regeneration of hepatocytes" has been suggested to be the morphological pathway to malignant transformation. HCV subtype Ib is the most prevalent in patients with HOC.

Studies of aflatoxin-DNA and aflatoxin-albumin adducts have greatly helped in confirming the carcinogenic role of this metabolite of *Aspergillus flavus* and so has the discovery of a G:C to T:A transversion at codon 249 of the p53 gene that appears to be specific for aflatoxin exposure in patients with HOC. Alcohol itself is not a carcinogen but it may act as a cofactor with viruses and chemicals and is, of course, a common cause of cirrhosis in Western countries. The carcinogenic effect of synthetic gonadal steroids is weak and, with doses currently employed, diminishing further.

Precancerous changes in the liver

Cirrhosis itself is a precancerous condition but the magnitude of the risk of malignant change varies with etiology, *i.e.,* high with HBV and HCV and low with alcohol. Large cell dysplasia (and small cell dysplasia) have been held to be associated with an increased risk of malignant change for many years, especially in studies from Africa, Italy and France, and macroregenerative nodule, adenomatous hyperplasia,. dysplastic nodule (synonyms denoting large cirrhotic nodules with cytological and architectural abnormalities) in Japan and the USA. Both suggestions have generated debate and neither can be regarded as a obligatory steps in carcinogenesis as they are not seen in noncirrhotic livers with HOC.

Pathology

Variations in the gross appearance of HOC (massive *vs.* multinodular) and histological grade (high *vs.* low) are not impressive between high and low incidence areas but are reflected, to some extent, in differences of clinical presentation (acute *vs.* chronic). The morphological classification promoted by WHO has stood the test of time. HOC reproduces the appearances of the normal liver with abnormal liver cell plates separated by sinusoids: this is a *sine qua non* requirement for diagnosis. The pattern may be further refined into purely trabecular, pseudoglandular (due to necrosis), compact (compression artefact) subtypes and, apart from the common liver-cell-like appearance, into clear cell and pleomorphic cell types. Mallory bodies, acidophilic globular inclusions and ground glass bodies may be seen. An important characteristic is deficiency or loss of the normal reticulin framework. Small (less than 2-3 cm) tumors and those that are (rarely) pedunculated, carry a much improved prognosis and so does fibrolamellar carcinoma, the only genuine subtype. The latter affect mainly young people of both sexes, is not associated with cirrhosis and less than 10% of patients show evidence of an etiology such as HBV or HCV.

Special techniques

Enzyme histochemistry and electron microscopy are seldom used nowadays but there has been an exponential growth in immunocytochemIstry. The cells of HOC elaborate many liver export proteins, such as AAT, albumin, ferritin, etc., but demonstration of bile canaliculi by polyclonal carcinoembryonic antigen (CEA) antisera is the simplest and most useful means of identifying a tumor as being of liver cell origin, Liver carcinoma cells elaborate cytokeratins 8 and 18, express major histocombatibilty complex, ABH and Lewis blood group antigens and show capillarization of sinusoids by positivity for 0D31 and 0D34 antigens, laminin and collagen type IV. The specificity of alpha-fetoprotein (AFP) production is high in the diagnosis of HOC but its sensitivity is low. The latest antigenic marker is HEP Par 1 for which a high liver cell specificity is claimed.

Studies of proliferative activity by AGNor count, proliferating cell nuclear antigen and MIB-1/Ki 67 expression have been correlated with growth, histological grade and prognosis. Ploidy by flow cytometry and morphometric analyses have also been used for the same purpose.

Loss of heterozygosity and mutations of p53 and Rb genes in human HOC have been found to be relatively late events and/or associated with poor prognostic parameters.

Prevention

Reduction of mortality by screening (ultrasound and AFP) for "early" small, treatable tumors has not been found to be universally successful and is expensive. Vaccination against HBV is effective and should greatly reduce the incidence of HOC in the future. In the meantime, millions remain infected with this virus and their outlook is grave: in an important follow up study from Taiwan, the relative death risk from HOC was calculated to be 98.4.

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