Hepatocellular Carcinoma Results from Chronic Cyclin D1 Overexpression in Transgenic Mice


ABSTRACT

Cyclin D1 is a known oncogene and a key regulator of cell cycle progression. Amplification of the cyclin D1 gene and its overexpression have been associated with aggressive forms of human hepatocellular carcinoma (HCC). In this study, two independent lines of transgenic mice have been generated that express cyclin D1 under the control of the rat liver fatty acid binding protein promoter. This transgene specifically directs expression in the liver and the intestines. RNA and protein analysis demonstrated increased expression of the cyclin D1 gene product in the liver and bowel when compared with wild-type siblings. Both transgenic lines developed progressive liver disease. Examination of H&E stained sections of the liver and bowel revealed hyperplastic changes in the liver by 3 months of age. By 6 months of age, transgenic mice had obvious hepatomegaly and histological evidence of dysplasia in the liver. These early changes were significantly more dramatic in male animals when compared with female animals. By 9 months of age adenomas of the liver appeared, progressing to HCC over the ensuing 6-month period. By 15–17 months of age, 87% of male and 69% of female animals had either adenomatous nodules or HCC. By 17 months of age, 31% of male and female animals had disease that had progressed to HCC. These animals represent a unique and significant new model for the study of human HCC. This study demonstrates that overexpression of cyclin D1 is sufficient to initiate hepatocellular carcinogenesis.

INTRODUCTION

The cyclin family of proteins is composed of highly conserved nuclear cell cycle regulatory proteins. The role of cyclin proteins in controlling the passage of cells through the cell cycle has been described in numerous experimental systems (1). D-type cyclin proteins accumulate during the G1 phase of the cell cycle and are exquisitely regulated by extracellular mitogenic signals (2–4). Transcriptional activation of the cyclin D1 gene occurs through the Ras signaling pathway via MAPKs (ERK) and ERK2 (5). In addition, experimental evidence shows that nuclear factor κB binding to the cyclin D1 gene promoter is critical for the regulation of cyclin D1 expression (6). In the absence of growth factor, cyclin proteins are rapidly degraded by a ubiquitin-proteasome-dependent mechanism (7, 8). When mitogenic stimulation is continuous and G1 cyclin levels are allowed to accumulate, then cyclins associate with their catalytic partners, cyclin-dependent kinases, to catalyze retinoblastoma phosphorylation thereby leading to progression of the cell cycle through the G1–S checkpoint. Among the cyclins, the D-type (D1, D2, and D3) is the primary regulator of and is absolutely required for cellular progression through the G1 phase of the cell cycle in cells with a functional retinoblastoma gene (9). Experimental evidence also shows that cyclin D1 expression is sufficient to promote hepatocellular cell cycle progression in the absence of mitogen (10). Thus, cyclin proteins in general and cyclin D1 in particular, serve as critical regulators of the cell cycle (11).

Translocation or amplification of the cyclin D1 gene (also known as the PRAD1 and bcl-1 oncogene) and subsequent overexpression have been described in a variety of human cancers (12). Cyclin D1 gene translocation resulting in increased cyclin D1 protein expression has been described for a subset of parathyroid adenomas (13) and in a subset of B-cell lymphomas (14). Overexpression of cyclin D1 protein is associated with poor prognosis in lung cancer (15) and HCC (16). Although cyclin D1 gene amplification is rare, increased cyclin D1 protein expression is common in colorectal cancer and its precursor, colorectal adenomatous polyps (17, 18). Cyclin D1 has been introduced as a transgene previously in experimental models of carcinogenesis with mixed results. In particular, overexpression of the cyclin D1 protein in breast tissue of transgenic animals resulted in mammary hyperplasia and adenocarcinoma in lactating mice (19). Targeted expression of a cyclin D1 transgene to the oral cavity and esophageal epithelium of mice led to dysplasia in the esophagus, but progression to esophageal carcinoma did not occur (20). Thus, although cyclin D1 behaves as an oncogene in some tissues, it has not performed as convincingly as an oncogene in others.

HCC is the most common form of malignancy in humans worldwide, representing 40% of all of the cancers in Southeast Asia, Japan, and Africa and 2–3% of all of the cancers in the United States (reviewed in Ref. 21). HCC is commonly associated with hepatitis B infection but also with liver cirrhosis, exposure to aflatoxin B, type I glycogen storage disease, α1-antitrypsin deficiency, and supplemental use of androgens. The disease is two to three times more prevalent in men than in women. It is frequently seen as both a unifocal as well as a multifocal disease. Lesions may appear as foci of well-differentiated, poorly differentiated or clear cell type hepatocytes. Currently, there are no adequate curative treatments for human HCC beyond resection for limited disease.

Recently, increased levels of cyclin D1 protein have been shown to be associated with aggressive forms of HCC (16, 22, 23). In addition, the cyclin D1 gene has been identified as a target gene in the Wnt signaling pathway as well as the Ras-activated MAPK signaling pathway, linking mutations that cause nuclear localization of β-catenin or MAPK/ERK1 activation to increased expression of cyclin D1 protein in a number of cancers including HCCs (24–26). It is not known whether increased cyclin D1 protein is a cause or a consequence of hepatic carcinogenesis. We hypothesized that targeted overexpression of cyclin D1 in the liver might be sufficient to cause hepatic tumor formation. Here we report the generation of two lines of transgenic mice in which a cyclin D1 transgene was expressed in the liver and bowel when compared with wild-type siblings. Both transgenic lines developed progressive liver disease. Examination of H&E stained sections of the liver and bowel revealed hyperplastic changes in the liver by 3 months of age. By 6 months of age, transgenic mice had obvious hepatomegaly and histological evidence of dysplasia in the liver. These early changes were significantly more dramatic in male animals when compared with female animals. By 9 months of age adenomas of the liver appeared, progressing to HCC over the ensuing 6-month period. By 15–17 months of age, 87% of male and 69% of female animals had either adenomatous nodules or HCC. By 17 months of age, 31% of male and female animals had disease that had progressed to HCC. These animals represent a unique and significant new model for the study of human HCC. This study demonstrates that overexpression of cyclin D1 is sufficient to initiate hepatocellular carcinogenesis.
ally lead to the development of multiple adenomatous lesions in the liver and ultimately to HCC with a high degree of penetrance. Thus, in independently derived transgenic lines, increased cyclin D1 expression led to hepatocyte transformation. These animals have the potential to serve as a valuable experimental model for HCC.

MATERIALS AND METHODS

Transgenic Mouse Production. The 600-bp rat LFABP promoter fragment (a gift from J. Gordon, Washington University, St. Louis, MO; Ref. 27) was ligated to the NotI site of the polylinker region of pEV containing the rabbit β-globin genomic sequence. We then ligated the 1.0-kb murine cyclin D1 cDNA sequence (a gift from C. J. Sherr, St. Jude Children’s Hospital, Memphis, TN; Ref. 4) cassette into the EcoRI site of exon II of the pEV β-globin. These manipulations produced a 3.0-kb transgene construct, excisable with XhoI, that was capable of directing cyclin D1 expression under the control of the rat LFABP promoter. Microinjection of single-cell C57BL6 mouse embryos was performed as described previously (28). At 3–4 weeks of age, mice were weaned, numbered, and tails were clipped for DNA analysis by PCR and Southern blot. Four transgenic founders were identified and bred to C57BL6 mice to establish the transgenic lines designated F1–F4.

DNA and RNA Analysis. DNA from short segments of mouse tail and RNA from tissue were isolated as described previously (29). Primers used for PCR of mouse tail DNA and RT-PCR of mouse organ-specific RNA were complementary to the cyclin D1 sequence 5′-AACAGATTGAAGCCCTTCTTCT-3′ and the 3′ end of exon II of the rabbit β-globin sequence 5′-ATCTCAGTTGATTTGTGA-3′ (Fig. 1A). For 30–35 cycles of PCR, nucleic acids were diluted in ddH2O to 8 ng/μl. Forty ng of nucleic acid was used per 50 μl reaction containing 10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl2, 0.01% (w/v) gelatin, 0.25 μM of each deoxynucleotidetriphosphate, 1.0 unit of AmpliTaq polymerase (PE Applied Biosystems, Inc., Foster City, CA), and 0.2 μM of each primer. Thermocycling was performed at 94°C for 1’, 55°C for 45’, and 72°C for 1’. For RT-PCR, an initial step at 48°C for 45’ followed by 94°C for 2’ preceded thermocycling at 94°C for 30’, 60°C for 1’, and 68°C for 2’. The PCR products were visualized on a 2.0% NuSieve 3:1 agarose (FMC BioProducts, Inc., Rockland, ME) gel in Tris-borate-EDTA with ethidium bromide. These manipulations produced a 3.0-kb transgene construct, excisable with XhoI, that was capable of directing cyclin D1 expression under the control of the rat LFABP promoter. Microinjection of single-cell C57BL6 mouse embryos was performed as described previously (28). At 3–4 weeks of age, mice were weaned, numbered, and tails were clipped for DNA analysis by PCR and Southern blot. Four transgenic founders were identified and bred to C57BL6 mice to establish the transgenic lines designated F1–F4.

RESULTS

Cyclin D1 cDNA was overexpressed in the liver and the intestines of transgenic mice using the −596 to +21 (EcoRI to PvuII) fragment of the rat LFABP promoter (Fig. 1A). This promoter directs expression exclusively to hepatocytes (excluding Kupffer and bile duct epithelial cells) primarily in the periportal zone (zone 1) of the liver. It also directs expression to villus-associated enterocytes of the small intestine and surface colonocytes of the large intestines (27). The presence of a 550-bp PCR product amplified by primers that span the junction of the cyclin D1 cDNA and rabbit β-globin sequences in the transgene identified these animals as transgene carriers. Carriers were confirmed by the presence of a 2.2-kb band on a Southern blot of Psrl-digested DNA probed with the cyclin D1 probe in all of the founder and breeder animal stocks (data not shown).

Western Blot Analysis. Rapidly prepared tissue lysates were prepared in standard lysis buffer (PBS containing 1% NP-40; 0.5% sodium deoxycholate; 0.1% SDS; and protease inhibitors phenylmethylsulfonyl fluoride, aprotinin, and Sodium Orthovanadate) on freshly retrieved tissues or from flash-frozen tissues. Protein concentrations of tissue lysates were determined by spectrophotometry (Bio-Rad Laboratories, Inc., Hercules, CA). Equal amounts (50 μg) of protein were resolved by SDS-PAGE and transferred to a 0.2 μm polyvinylidene difluoride using a semidry transfer apparatus. Membranes were incubated overnight at room temperature in blocking solution (Tris-buffered saline containing 5% nonfat dried milk and 0.05% NaNO3 and 0.1% Tween 20) before probing 2 h with polyclonal rabbit anti-Cyclin D1 antibody (Upstate Biotech, Inc., Lake Placid, NY, Cat. No. 06137) in blocking solution (diluted 1:100). Filters were then washed 3 times and incubated with horseradish peroxidase-conjugated goat antirabbit IgG (1:1000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for 1 h. Positive bands were detected by the enhanced chemiluminescence system (Amersham-Pharmacia Biotech, Inc., Piscataway, NJ).

Histology and Immunohistochemistry. Freshly dissected tissues from animals were taken from animals sacrificed within 2 h of the fasting midpoint (10 a.m.–2 p.m.). Tissues were divided and one part fixed in 4% paraformaldehyde (Sigma Chemical Co., St. Louis, MO) for 4–8 h then transferred to 70% ethanol. Fixed tissues were embedded in paraffin. Sections (5 μm) were stained with H&E before analysis.

Liver sections were graded in a blinded analysis by a pathologist (K. W.). Criteria for the designation of hyperplasia included the presence of cells with enlarged and irregular nuclei and frequent mitotic and apoptotic bodies. Criteria for designation of dysplasia included in addition a threshold for hepatocellular nuclear size >20 μm in ≥5 cells/high-powered field (>400). HCA and HCC were scored based on the criteria of Frith and Ward (30). Briefly, well-circumscribed expanding nodules ≤5 mm in diameter made up of cells with a uniform size and appearance were designated HCA, whereas larger nodules (>5 mm) with a distinct trabecular cellular architecture were designated as HCC.

For immunohistochemistry, 5 μm sections were deparaffinized with xylene, rehydrated in a graded series of ethanol, and carried into PBS. Endogenous peroxidase activity was quenched in 3% H2O2 followed by an alkaline antigen retrieval step (pH 10.0; Biogenix, San Ramon, CA). Additional nonspecific background staining was blocked using the MOM peroxidase-based kit (Vector Laboratories, Burlingame, CA). The primary antibody used was a monoclonal mouse anti-human cyclin D1 antibody (Santa Cruz Biotechnology, Inc.) used at a dilution of 1:20. Amplification of the reaction was achieved by incubating the sections with the Elite Avidin-Biotin kit supplied by Vector labs. Sites of immunoreactivity were visualized using 3,3′-diaminobenzidine as the chromogen (DAKO Co., Carpinteria, CA). Slides were viewed under a Zeiss Axioplan 2 microscope, and images were captured using a model HRP042-CMT digital camera from Diagnostic Instruments and Zeiss Image 3.0 software. Images were processed using Adobe Photoshop software (Adobe Systems Inc., San Jose, CA) and printed on a Tektronix Phaser 450 color printer.

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Fig. 1. Transgene construct, integration, and expression. A, schematic showing the murine cyclin D1 cDNA (1.1 kb), inserted into exon II of rabbit β-globin gene and driven by the 0.6-kb LFABP promoter. Transgene-specific PCR primers span the CcnD1/β-globin junction. B, cyclin D1 protein levels analyzed by Western blot in various organs of control and F4 transgenic male animals at 1 month of age. Lane 1, heart; Lane 2, lung; Lane 3, liver; Lane 4, spleen; Lane 5, small intestine; Lane 6, large intestine; Lane 7, kidney; Lane 8, testes. CcnD1 band is M, 36,000. Equivalent protein load confirmed by actin.

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faithfully transmitted through the germ line to subsequent generations. As estimated by the densitometric measurement of the intensity of transgene specific bands on Southern blots, the F4 line carried ~20 times the number of copies of the transgene as the F2 line (data not shown). Animals derived from the F4 founder mouse expressed an overabundance of cyclin D1 protein in the liver as well as in the small and large bowel and to a lesser extent in the kidney (Fig. 1B). On the basis of equal loading of protein gels, the F4 line expressed three to four times the amount of cyclin D1 protein in the liver as compared with the F2 line. Overexpression of cyclin D1 protein in the small and large bowel was not detected in the F2 line. All of the experimental animals were identified as carriers or controls by PCR, and protein expression was confirmed by Western blot analysis.

At each of the 6 time points, animals were sacrificed and autopsied for differences in body weight and target tissue weight. There was no trend toward significant differences in body weight between transgenic and wild-type siblings throughout the experimental period (data not shown). Although transgene expression remained high in the small and large intestines of the F4 animals throughout the experimental period, no pathological consequences were observed either grossly or histologically in these tissues. Cyclin D1 protein levels in target tissues remained elevated as compared with control littermates from weaning through 17 months of age. These elevated cyclin D1 levels were accompanied by progressive phenotypic changes in the liver.

**Cyclin D1 Overexpression Is Associated with Gender-dependent Hepatomegaly and Hepatocellular Changes.** By 1 month of age and continuing through 12 months of age, hepatomegaly (defined here as an increase in liver:body weight ratio of ≥10%) developed in all of the transgenic animals, increasing over the time course of the experiment. Hepatomegaly was also most pronounced in the F4 line and was significantly more severe in male animals when compared with female animals at all of the time points (P < 0.001 in a multivariant analysis; Fig. 2A). At this time point and throughout the remainder of the experimental period, there was no significant difference in the level of cyclin D1 protein expression between male and female animals (data not shown). Differences in the extent of hepatomegaly between F2 and F4 animals were also noted, though these differences were not found to be statistically significant over the time course (P = 0.272; Fig. 2B).

Frequent mitotic bodies and dysplastic cell nuclei were evident in transgenic liver sections over and above that found in control-matched sibling liver sections through the first 6 months of observation. In control livers, mitotic figures had disappeared after 1 month of age, and dysplastic cell nuclei were nonexistent. Quantitation of mitotic bodies in transgenic liver sections demonstrated a significantly higher rate of mitosis in male versus female animals over time in a multivariant analysis (P = 0.020). Similarly, dysplastic cell nuclei (those measuring ≥20 μm) were also significantly more numerous in male versus female animals in a multivariant analysis (P = 0.002). Nuclear shapes were highly irregular, forming notched shapes, slipper shapes, and in some cases a dumbbell shape that seemed to suggest incomplete mitosis. Tripolar mitotic bodies were also evident. Some abnormally large hepatocytes contained large nucleoli, nuclear and cytoplasmic inclusions, glycogen bodies, and clumped cytoplasmic material suggestive of Mallory’s hyaline. Hepatocytes in zones 1 and 2 were slightly smaller than normal, with a slightly increased nuclear:cytoplasmin ratio.

By 6 months of age, 100% of the F4 male animals and 25% of the F4 female animals had liver changes that were consistent with dysplasia (Fig. 2C). Designation of dysplasia involved the appearance of cellular changes such as a baseline level of meganuclei and number of aberrant mitotic figures found in H&E-stained liver sections. The F2 line animals showed a pattern of disease progression similar to the F4 line with delayed kinetics (Fig. 2D). By this later time point, disease had progressed in both male and female animals to HCA as described below.

Light microscopy of H&E-stained sections revealed that hepatocellular dysplasia was distributed primarily in the zones surrounding central veins (zone 3) in transgenic livers. By 3 and 6 months of age, the centrilobular zone of altered hepatocytes had become progres-
sively larger and more distinct. Nuclear changes in zone 3 hepatocytes were also more pronounced (Fig. 3, A and B). Larger nuclei were hyperchromatic with coarsely clumped chromatin and were lobulated and irregular in outline. Liver sections from both transgenic lines at 3 months of age showed cyclin D1-specific nuclear immunoreactivity. In contrast, there was no detectable cyclin D1-specific immunoreactivity in the same tissues of wild-type age-matched sibling control animals (Fig. 3C). Cyclin D1-specific nuclear staining was distributed uniformly throughout the livers of transgenic animals comprising 80% of hepatocytes (Fig. 3D). There was no demonstrable difference in the intensity or topography of cyclin D1 staining between male and female animals (data not shown).

Apoptosis in Transgenic Livers and the Development of Nodular Lesions Is a Gender-independent Phenomenon in LFABP-Cyclin D1 Animals. Examination of H&E-stained transgenic liver sections revealed a high number of apoptotic bodies through the 6th month of observation relative to control livers in which apoptotic bodies were rarely detected. Interestingly, apoptotic counts over time in male versus female transgenic livers were statistically similar in a multivariate analysis (P = 0.719; Fig. 4A). At 9 and 12 months of age, the number of apoptotic bodies detected in transgenic livers declined slightly but persisted above the number detected in controls (data not shown).

By 9 months of age, the first nodular foci of poorly differentiated hepatocytes were observed in the liver of an F4 male animal. By 12 months, multifocal eosinophilic and clear cell nodules were apparent in both F4 male (four of seven) and F2 male (three of eight) and in F4 female (two of four) but not F2 female (zero of two) animals. Up to four to seven nodules could be found on the surface individual livers from transgenic mice by 12 months of age. Nodules typically ranged in size from 1–2 mm in diameter. Whereas the overall trend toward development of hepatic disease (including hepatomegaly and dysplasia) was significantly different between male and female transgenic mice in a multivariate analysis (P = 0.008 for the F2 line and P = 0.017 for the F4 line), differences in similar comparisons looking at emerging expanding lesions were statistically similar between genders (P = 0.940 for F2 and P = 0.246 for F4; Fig. 4B).

Histologically, the liver nodules were made up of groups of cells that were smaller than normal hepatocytes and were well circumscribed (Fig. 5A). Cells within the nodules stained positive for cyclin D1 (Fig. 5B). In many areas, these small cell nodules lay contrasted against a backdrop of larger, dysplastic cells. Nodule size as well as the absence of a trabecular structure was used to classify these early lesions as adenomas by the criteria of Frith and Ward (30).

By 17 months of age, all transgenic animals had progressed from mild to severe hepatomegaly (50% increase in liver:body weight ratio over controls for F2 and F4 males, 23% for F4 females). Hepatocellular lesions had progressed to include an increasing fraction of nodules in various stages of development from adenoma to HCC (Fig. 5, C and D). Both cells within the HCC lesions and in the adjacent tissue characteristically stained positive for cyclin D1 protein (Fig. 5, E and F). Although significant background staining was frequently seen in areas of nodular lesions, this finding was not consistent enough to be attributed to the phenotype. RT-PCR analysis confirmed the persistent expression of the transgene in the area of the tumor as well as in the adjacent tissue (Fig. 5G).

Thirty-three % of F2 animals examined (three of nine total animals), had expanding lesions in their livers, none of which had advanced to HCC. Eighty-seven % of the F4 male and 69% of the F4 female animals at 17 months of age exhibited lesions of various stages including adenoma, HCC within an adenoma, and frank HCC (Table 1). Among 13 F4 female animals, 9 (69%) animals had nodular lesions, 4 (31%) of those representing HCC. Similarly among 16 male animals, 14 (87%) animals had lesions of which 5 (31%) represented

Fig. 3. Transgenic livers exhibit cellular changes in the perportal zone. H&E-stained transgenic liver sections from 6-month-old wild-type (A) and F4 transgenic (B) male animals at 3 months of age are shown at ×200; bar, 0.25 mm. Arrows, central veins and dysplasia in zone 3 of B. Block arrows, portal triads. Cyclin D1 immunostain of wild-type (C) and F4 transgenic (D) animals at ×400; bar, 0.12 mm. Arrows, central veins.
HCC. Measurable lesions ranged in size from 0.6 mm to 1.7 cm. There was no significant difference in the size of lesions measured in male as compared with female mice. Two lesions, one in a female transgenic liver and another in a male transgenic liver, represented a transitional HCC focus within an adenomatous lesion. Overall, 76 lesions were counted and measured in 29 F4 animals between 9 and 17 months of age. None of the control sibling littermates developed adenomas or carcinomas of the liver.

Throughout the experimental period, transgenic mice showed no changes in cell architecture within the small and large bowel. No significant differences in measurements of the crypt:villus ratio were detected (data not shown). No epithelial nodule development, gut hyperplasia, or aberrant crypt foci were found in any of the animals used in the study.

**DISCUSSION**

In the present study, we describe two lines of transgenic mice, each one overexpressing the cell-cycle regulatory gene, *cyclin D1*, under the control of the LFABP promoter. These independent lines have distinct transgene integration properties as demonstrated on Southern blot analysis and as reflected by identifiable differences in transgene expression. The two transgenic lines develop liver disease in parallel with one another, differing only slightly in the kinetics of disease progression. These differences are likely attributable to a gene dosage effect in the liver. Our results indicate that the increased expression of *cyclin D1* in both transgenic lines is sufficient to initiate hepatic neoplasia with histopathological characteristics resembling human HCC. Progression of disease accompanied by *cyclin D1* overexpression mirrors what is known about the progression of 10–13% of HCCs in humans. The stochastic appearance of the liver tumors strongly suggests that overexpression of *cyclin D1* as an initiating event is conducive to the development of additional genetic lesions that enable progression from hyperplasia to HCC. Therefore, these animals represent a unique and significant model for the study of prevention and treatment of HCC.

In *cyclin D1* transgenic mice, hepatic adenomas and HCC was...
preceded by gender-dependent liver cell dysplasia in centrilobular (zone 3) areas. This gender-dependent phenomenon was determined not to be attributable to a gene dosage effect. Concomitant with dysplasia was a relatively high rate of cell division. Zone 3 dysplasia is commonly seen in other transgenic liver disease models (SV40 TAg, HBV, TGF-α, c-myc) and has been extensively reviewed (32, 33). Characteristics of the dysplastic phenotype include cellular and nuclear pleomorphism and hypertrophy, multiple prominent nucleoli, and nuclear pseudo-inclusions. Frequent aberrant mitotic figures in dysplastic cells are typically observed. We observed dysplasia in transgenic livers that was proportionate in severity to the degree of transgene expression in F2 versus F4 animals. Dysplastic cells in the c-myc/TGF-α model, also in the pericentral zone, have been shown to produce abundant TGF-β1 and undergo apoptosis, presumably as an autocrine mechanism of protection against unscheduled and aberrant cellular proliferation (34). Thus, the dysplastic cells in zone 3 are not considered to be good candidate cells for clonal expansion. Similarly, the tumors arising in the cyclin D1 transgenic mice do not appear to be directly linked to or to arise from the dysplastic cell type. First, the gender-independent frequency of the transformation is distinct from the gender-dependent frequency of the dysplastic cell type. Second, hepatocytes within the focal nodule are much smaller, more uniform in size and shape, and have a higher nuclear:cytoplasmic ratio than the dysplastic cells. It has been suggested that periporal cells respond to the death of cells in zone 3 by overcompensating with proliferation, resulting in multifocal hyperplasia and hepatomegaly (35, 36). In fact, the LFABP promoter has been shown to express at its highest level in the periporal zone (27). Additional genetic mutations, brought about by multiple rounds of proliferation in cells that are outside of zone 3, are thought to bring about eventual transformation among cells in this population.

The increased severity of early disease in male animals was associated with the degree of hepatomegaly, mitotic activity in the liver, and the development of aberrant hepatocytes. These features were only partially abrogated by castration at 4 weeks of age (data not shown). Interestingly, the degree of apoptosis occurring in the liver was the only parameter measured early on in disease progression that was not gender dependent. This feature has a stronger correlation with the ultimate development of HCA or HCC (87% male versus 69% female), which was not statistically significant (P = 0.246). These observations suggest that the degree of cellular transformation is linked to the apoptosis in these livers. This apparent linkage may be related to an increased expression of TGF-β1 and growth arrest, and apoptosis associated with increased ubiquitin-dependent cyclin D1 degradation. J. Biol. Chem., 275: 22916–22924, 2000. 9. Baldin, V., Lukas, J., Marcote, M. J., Pagano, M., and Draetta, G. Cyclin D1 is a nuclear protein required for cell cycle progression in G1. Genes Dev., 7: 812–821, 1993.


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