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Evolution of Neoplastic Development in the Liver of Transgenic Mice Co-Expressing c-*myc* and Transforming Growth Factor-α

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We have previously shown that co-expression of c-myc and transforming growth factor (TGF)-α as transgenes in mouse liver results in major enhancement of neoplastic development in this organ as compared with expression of either of these transgenes alone. In this report we describe in detail the progression from liver cell dysplasia to hepatocellular carcinomas (HCCs) occurring in the liver of c-myc/TGF-α and c-myc transgenic mice. Despite morphological similarities in the sequence of events between the two transgenic lines, the dramatic acceleration, extent, and severity of hepatic lesions in c-myc/TGF-α mice clearly demonstrated the synergistic effects of this transgenic combination. Although c-myc/TGF-α and c-myc females displayed longer latency and lower tumor incidence, the pathological changes were the same as those seen in the male mice, including the formation of HCCs, which are absent in TGF-α single-transgenic females. Tumors in single- and double-transgenic mice showed induction of the endogenous c-myc and TGF-α and, most frequently, unchanged or decreased epidermal growth factor receptor, further indicating the collaborative role of c-myc and TGF-α in providing a selective growth advantage to tumor cells independently of the epidermal growth factor receptor levels. To identify possible tumor precursors, we focused particularly on the dysplastic changes preceding and accompanying the appearance of preneoplastic and neoplastic lesions in the double-transgenic mice. Early on, these changes were characterized by the appearance of large dysplastic hepatocytes, mostly pericentrally, expressing high levels of TGF-α and uPA, as well as TGF-β1, particularly in apoptotic cells. After a short period of replication and expansion into the liver parenchyma, as well as penetration into the central veins, these cells underwent apoptotic cell death while preneoplastic and neoplastic lesions were forming. The peritumorous tissues also contained small dysplastic hepatocytes and oval-like cells, similar to those found in the tumors. Transplantation of the transgenic liver tissues harboring only dysplasia with or without vascular lesions onto nude mice was able to yield HCCs composed of small diploid cells, suggesting that initiated cells are generated during the early dysplastic phase and can progress to HCC. It is therefore likely that large dysplastic hepatocytes undergo apoptosis, which may be closely associated with the up-regulation of TGF-β1 and uPA, whereas other cells evolve into the precursor population for HCC. Due to the simultaneous presence of c-myc, TGF-α, and dysplasia in premalignant human liver diseases, our transgenic mouse system appears to be an appropriate model for studying human hepatocarcinogenesis. (Am J Pathol 1996, 149:407–428)

In light of the central role that c-*myc* plays in cell replication, differentiation, and apoptosis,[1] it is not surprising that aberrant expression of c-*myc* has been implicated in the development of a wide variety of both experimentally induced and naturally occurring tumors,[2] including hepatocellular carcinoma (HCC).[3–6] Several other oncogenes and growth factors are known to interact with members of the c-*myc* family during neoplastic development, and this interaction may be critical in the evolution of the malig-

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nant phenotype.[7] We have recently established a transgenic mouse model aimed at exploring the interaction of nuclear oncogenes such as c-*myc* and growth factors acting via tyrosine kinase receptors.[8] In this model we have shown that co-expression of c-*myc* and transforming growth factor (TGF)-α as transgenes in the mouse liver results in greatly accelerated neoplastic development in this organ as compared with either of these oncogenes alone.[8] Similar cooperation between c-*myc* and TGF-α or c-*myc* and epidermal growth factor (EGF) in accelerating hepatocarcinogenesis has also been shown in transgenic mice models by Sandgren et al[9] and Tönjes et al,[10] respectively.

Earlier work by Sandgren et al[11] showed that transgenic mice bearing a fusion gene consisting of albumin enhancer/promoter c-*myc* cDNA (Alb-c-*myc*) display diffuse dysplastic changes and later hepatocellular adenomas (HCAs) developed in the liver, but no carcinomas were observed in these animals before 18 months of age. As shown in the present report, development of HCCs occurred in our Alb-c-*myc* single-transgenic mice within the age of 12 to 15 months. It has been demonstrated that livers of transgenic mice bearing a metallothionein promoter (MT) driven TGF-α transgene developed hyperplastic changes[12,13] followed by the appearance of tumors usually at 10 to 15 months of age.[13,14] In contrast, the process of hepatocarcinogenesis is accelerated in the c-*myc*/TGF-α double-transgenic mice and results in the formation of carcinomas as early as 4 months. Interestingly, the synergistic effect of c-*myc* and TGF-α in tumorigenesis appears to be very potent also in transgenic mouse models of other glandular organs, such as pancreas, mammary gland, and salivary glands.[9,15]

In the present study we provide an extensive and detailed analysis of the pathological changes sequentially occurring during liver oncogenesis in the double-transgenic c-*myc*/TGF-α and single-transgenic c-*myc* mice that we had not previously reported. We place special emphasis on the marked dysplastic changes preceding the emergence of carcinomas and the potential relationship of these lesions to the generation of precursors for liver tumors. As c-*myc* has been suggested to sensitize liver epithelial cells to growth factors[16,17] and possibly facilitate tumorigenic transformation by a TGF-α/epidermal growth factor receptor (EGF-R) autocrine growth mechanism,[18] we have analyzed the expression of the transgenes, their endogenous counterparts, and the EGF-R in dysplastic nontumorous and in tumorous tissues of our transgenic mice.

The results indicate that the cooperation between c-*myc* and TGF-α, either as transgenes or as endogenous genes, is a common event characterizing liver tumor progression. Furthermore, we show that double-transgenic c-*myc*/TGF-α mice represent an interesting model for examining the relationship between early high grade dysplasia and subsequent HCC, an issue that remains still controversial in experimental and human hepatocarcinogenesis. The dysplastic cell population in the transgenic mice is composed mostly of enlarged and damaged hepatocytes, which after a period of abnormal replication undergo apoptosis induced, at least in part, by overexpression and activation of TGF-β1. Transplantation of the transgenic liver tissues harboring only dysplasia onto nude mice was able to yield HCCs, implying that the initiated cells are generated during the early dysplastic phase and can progress to HCC.

Materials and Methods

Construction of Fusion Genes and Generation of Transgenic Mice

Generation of the Alb-c-*myc* (c-*myc*) single-transgenic and Alb-c-*myc*/MT-TGF-α (c-*myc*/TGF-α) double-transgenic mice was achieved as described earlier using (C57BL/6JXCBA/J)F1 and ((C57BL/6JXCBA/J)XCD1)F1 mice, respectively.[8] The construction of the transgenes has been described previously.[8,13] Transgene screening was performed by Southern blot analysis of the tail DNA after appropriate restriction nuclease digestion. The double-transgenic mice were maintained on 50 mmol/L $ZnCl_2$ in the drinking water starting at 3 weeks of age to enhance the transgenic TGF-α expression. Animal housing and care were in accordance with National Institutes of Health guidelines.

Gross and Histopathological Analyses

Ten to twelve male and three to five female c-*myc*/TGF-α double-transgenic mice were sacrificed every month until they were 12 months old. At this age, the double-transgenic animals succumbed at a high rate or were moribund, so that their examination afterwards was no longer possible. Ten to twelve male and three to five female c-*myc* single-transgenic mice, as well as three male and three female wild-type mice were sacrificed every month until they were 12 months old, and thereafter every second month until the age of 20 months. Body weights were recorded and livers were obtained by autopsy,

weighed, and examined for gross lesions. Specimens from each lobe and from tumors ≥3 mm were fixed in 10% buffered formaldehyde, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E), periodic acid-Schiff (PAS), glucose 6-phosphatase, and Masson's trichrome, all according to standard methods. For molecular analysis, nontumorous tissues and individual tumors were quickly frozen in liquid nitrogen and stored at −80°C until use. Histopathological diagnoses were based upon criteria described by Frith et al[19] and independently performed by two different investigators (E. Santoni-Rugiu and P. Nagy).

Transplantation of Dysplastic Liver Tissue

Approximately 3 × 3 × 3 mm pieces of freshly removed livers from 1.5-, 2.5-, and 5-month-old c-*myc*/TGF-α transgenic mice were aseptically transplanted under the skin on both sides of nude mice. Extensive histological examination was performed in each case on the nontransplanted part of the liver. The nude mice were observed for 3 months, and subcutaneous tumors were removed for further analysis.

Gene Expression and Immunohistochemistry

In situ hybridization (ISH), RNA isolation, and Northern blot analysis were performed as described earlier using radioactive probes.[6] The ISH for TGF-β1 was also performed by using a nonradioactive digoxigenin-labeled RNA probe according to the manufacturer's instructions (Boehringer Mannheim, Indianapolis, IN). For ISH, mouse α-fetoprotein (AFP) probe (kindly provided by Dr. S. Tilghman, Yale University, New Haven, CT) and the human TGF-β1 (clones phTGFB-2, purchased from American Type Culture Collection, Rockville, MD) were used. Northern analysis was carried out by using the following probes: a 316-bp *Eco*RI fragment of rat TGF-α cDNA, a mouse c-*myc* cDNA probe previously described,[8] a 2.2-kb *Eco*RI fragment of the rat EGF-R cDNA, a 400-bp *Pst*I-*Sma*I fragment of the mouse uPA cDNA (a gift of Dr. J. L. Degan, University of Cincinnati, Cincinnati, OH), and an 847-bp fragment of the human hepatocyte growth factor coding region (kindly provided by Dr. R. Zarnegar, Department of Pathology, University of Pittsburgh School of Medicine, Pittsburgh, PA). A 510-bp fragment of rat albumin cDNA was utilized to normalize the expression levels of the other genes. The Northern blots were exposed to Kodak XAR film (Eastman Kodak Co., Rochester, NY) at −80°C and also analyzed by phosphorimager to quantify mRNA expression using ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

With the exception of A6 immunohistochemistry, all of the immunohistochemical stainings were performed on paraffin sections. After deparaffinization and blocking of the endogenous peroxidase activity by standard methods, the sections were incubated with the primary antibody (Ab) in phosphate-buffered saline (PBS) overnight at 4°C. The binding of the Ab was revealed utilizing the appropriate Elite Vectastain ABC kit (Vector Laboratories, Burlingame, CA) and diaminobenzidine as peroxidase substrate. To localize active TGF-β1, the rabbit polyclonal LC(1–30) Ab (kindly provided by Drs. Kathleen Flanders and Anita Roberts, NCI, Bethesda, MD) against the amino-terminal 30 amino acids of mature TGF-β1,[20] was utilized. This Ab produces an intracellular staining on formalin-fixed, paraffin-embedded tissues.[21] Rabbit polyclonal Ab for mouse uPA was purchased from American Diagnostica (Greenwich, CT), whereas mouse monoclonal anti-proliferating cell nuclear antigen (anti-PCNA) Ab was from Dako Corp. (Carpinteria, CA). The rabbit polyclonal TGF-α Ab raised against residues 137 to 159 in the carboxyl-terminal cytoplasmic domain of rat pro-TGF-α[22] was a gift from Dr. L. Gentry (Medical College of Ohio, Toledo, OH). As negative controls, tissue sections were incubated in PBS containing rabbit and mouse serum without primary Ab.

Immunostaining with monoclonal A6 Ab that recognizes common surface-exposed antigens of mouse biliary epithelial cells and oval cells was performed on frozen and on paraffin sections as previously described.[23]

Immunoblot Analysis

For Western blot analysis of mouse EGF-R, liver samples were homogenized in lysis buffer containing protease and phosphatase inhibitors and sonicated. One hundred micrograms of proteins solubilized in boiling Laemmli buffer containing 2β-mercaptoethanol were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. The blots were probed with a rabbit polyclonal Ab against the cytoplasmic domain of human EGF-R (Santa Cruz Biotechnology, Santa Cruz, CA). The reaction was revealed by an ECL kit according to the manufacturer's instructions (Amersham, Arlington Heights, IL).

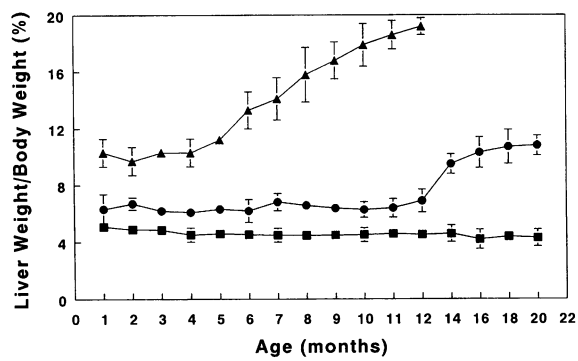

Figure 1. *Liver weight in wild-type* (■), *c*-myc *single-transgenic* (●), *and c*-myc/*TGF-α double-transgenic* (▲) *mice. Values are expressed as percentage of total body weight ± SE.*

Results

Changes of Liver Weights in c-myc and c-myc/TGF-α Transgenic Mice

The three c-*myc* transgenic mouse lines ((C57BL/6JXCBA/J)F1) expressing high levels of transgene in the liver (lines 166.8, 178.3, and 181.2)[8] have all been successfully mated with the TGF-α transgenic mouse line MT42.[13] As no morphological differences were observed among the three single-transgenic lines and among the three resultant double-transgenic ones, we have combined the data for the present analysis. In addition, the single-transgenic c-*myc* mice displayed the same phenotype in a different background ((C57BL/6JXCBA/J)XFVB) generated for a separate study.[81]

The ratio of liver weight to body weight provided the first indication of the abnormal liver growth and neoplastic development in the transgenic animals (Figure 1). During the first year, the liver weight/body weight ratio of c-*myc* single-transgenic mice was approximately 1.3 to 1.5 times higher than the ratio in wild-type animals. The highest values in c-*myc* transgenic mice were reached at the ages of 2 months and 7 months, which correspond to the completion of juvenile liver growth and to the appearance of preneoplastic lesions (Figure 2), respectively. There-

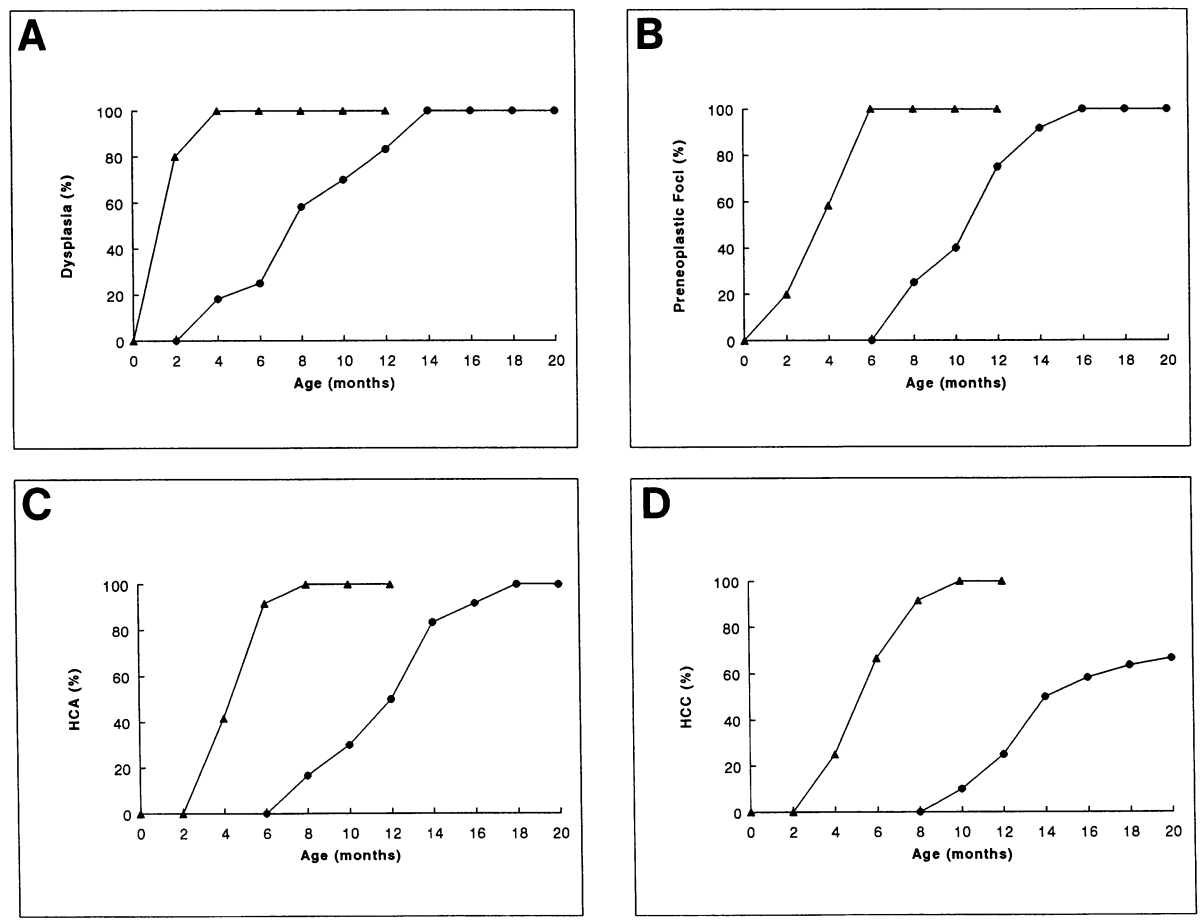

Figure 2. *Incidence and time course of hepatic lesions in c*-myc (●) *and c*-myc/*TGF-α* (▲) *transgenic mice.* A: *Dysplasia.* B: *Preneoplastic foci.* C: *HCA.* D: *HCC. Values are percentages of animals affected at each time point.*

Table 1. *Frequency (Percentage) of Lesions in Male and Female Transgenic Mice*

Age (months)	Sex	Number of mice	Dysplasia	Foci	Adenoma	Carcinoma	Blood vessel invasion*
c-*myc*/TGF-α							
0–2	F	8	25	0	0	0	0
	M	20	80	10	0	0	0
2–4	F	6	100	0	0	0	0
	M	24	100	58.3	33.3	12.5	46.8
4–6	F	6	100	66.7	0	0	16.7
	M	24	100	100	91.7	62.5	29.2
6–8	F	10	100	40	20	30	10
	M	24	100	100	100	100	0
8–10	F	10	100	40	20	30	10
	M	24	100	100	100	100	0
c-*myc*							
10–12	F	8	37.5	2.3	25	0	0
	M	22	77.3	59.1	40.9	18.2	0
12–14	F	10	60	50	40	0	0
	M	24	91.7	83.3	66.7	37.5	0
14–16	F	10	80	60	40	10	0
	M	24	100	95.8	91.7	54.2	0
16–18	F	10	90	70	20	0	0
	M	23	100	100	95.6	0	0
18–20	F	10	100	80	30	10	0
	M	23	100	100	100	65.2	0

F, female; M, male.
*Only by dysplastic cells.

fore, in contrast to the TGF-α single-transgenic mice,[24,25] the c-*myc* mice showed during the first 12 months no apparent decline of the liver weight relative to total body weight. This observation suggested that c-*myc* overexpression in hepatocytes prevents the correction of the altered functional mass (reviewed in Ref. 26) by compensating cell turnover with a continuously high cell proliferation until the appearance of neoplastic lesions. When neoplastic lesions developed, the liver weight/body weight ratio of c-*myc* animals increased significantly, reaching, after 16 months of age, values approximately 2.5 times higher than in control mice (10.8 ± 0.7% *versus* 4.3 ± 0.4% at 20 months of age).

The liver weight/body weight ratio of young c-*myc*/TGF-α double-transgenic mice was higher than the ratio in either of the single-transgenic mice, indicating a considerable preneoplastic liver enlargement due to hepatocyte hypertrophy and hyperplasia caused by co-expression of both transgenes. Similar levels of liver enlargement before the occurrence of neoplastic lesions were observed in TGF-α transgenic mice treated with phenobarbital.[24] After 4 months of age, the appearance of large tumor masses that gradually replaced the entire hepatic parenchyma and invaded the abdominal cavity and the consequent malnutrition resulted in very high liver weight/body weight ratios in c-*myc*/TGF-α mice. Thus, in the double-transgenic animals capable of surviving up to 12 months, the liver weight represented nearly 20% of body weight (Figure 1).

Development of Hepatic Neoplasia in c-myc *and* c-myc/TGF-α *Transgenic Mice*

The incidence and time course of the major morphological changes are summarized in Figure 2 and in Table 1. Most of the c-*myc*/TGF-α double-transgenic mice sacrificed within the first 2 months had already extensive dysplastic changes in the liver (Figures 2A and 3A). The extent and severity of hepatocyte dysplasia ranged from moderate cellular and nuclear pleomorphism and hypertrophy, multiple prominent nucleoli, frequent mitoses, nuclear eosinophilic pseudoinclusions, and intranuclear lipid droplets (Figure 3B) to a more advanced polymorphism with severe cytomegaly and karyomegaly and multinucleation combined with abnormal mitotic figures. These included tripolar mitoses, chromosomal bridges, and aberrant chromosomal migration, all indicating severe DNA damage (Figure 3C). The dysplastic changes started in the perivascular areas of the liver and were most advanced around the central veins. By the second month, the dysplasia had quickly spread throughout the hepatic lobule affecting the periportal zone, although the basic lobular structure was preserved (Figure 3A). Apoptotic cells were also present in dysplastic areas, mostly represented by

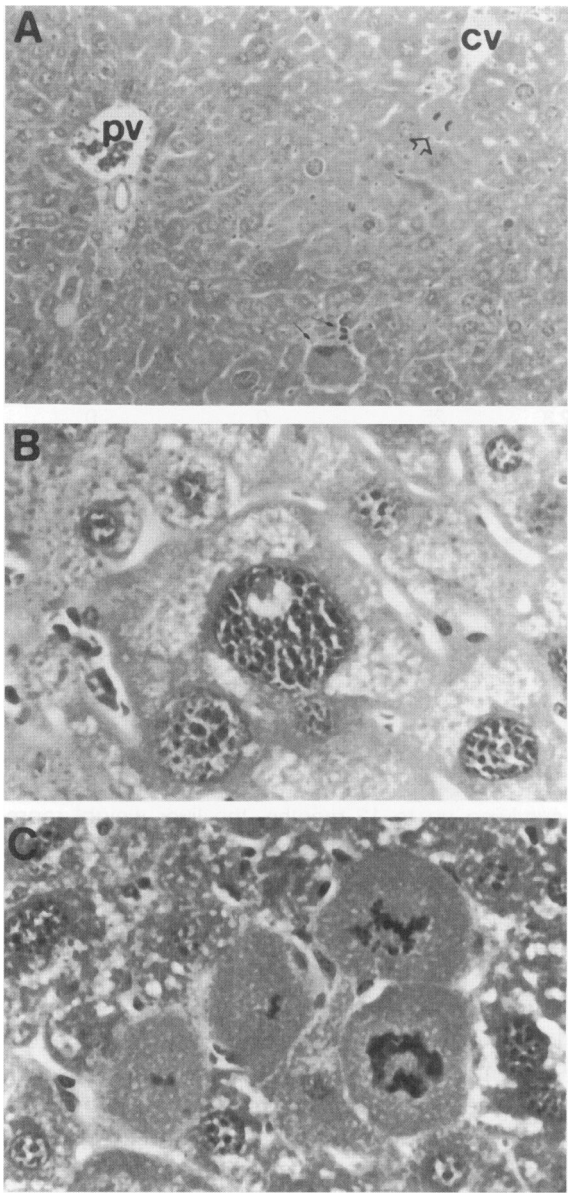

Figure 3. *Patterns of dysplastic lesions in c-myc/TGF-α transgenic mice.* A: *Widespread liver dysplasia in a 2-month-old male animal. Some dysplastic hepatocytes are undergoing apoptosis* (thin arrows) *or mitosis* (open arrow). *pv, portal vein; cv, central vein.* B: *Typical large dysplastic hepatocyte characterized by cellular and nuclear pleomorphism and hypertrophy, multiple prominent nucleoli, and nuclear pseudoinclusions.* C: *Atypical mitoses in dysplastic hepatocytes. H&E; magnification, ×150* (A) *and ×2000* (B *and* C).

large dysplastic hepatocytes with condensed and homogeneously stained nuclei, apoptotic bodies, eosinophilic cytoplasms, and usually an empty space between neighboring cells (Figure 3A). Sometimes focal coagulative necrosis of hepatocyte groups with granulocytic reaction was also detected.

The diffuse dysplastic changes described above were present in the livers of all of the double-transgenic mice after 2 months of age, when focal lesions (foci of cellular alteration) were also present in most of the male animals (Figure 2B and Table 1). The number of preneoplastic foci was very variable, but morphologically these lesions were similar to the hepatic foci produced in chemical hepatocarcinogenesis protocols. Clear-cell foci (Figure 4A) were the most frequent type, but basophilic (Figure 4B), eosinophilic, and mixed types of foci also occurred. The cells of the foci did not show much variability, in striking contrast with the surrounding dysplastic liver. The first benign (HCAs) and malignant (HCCs) neoplastic lesions appeared in c-*myc*/TGF-α transgenic mice between the third and fourth month, and by the eighth month, 100% of the males and 30% of the females had liver carcinomas in a background of hepatic dysplasia (Figure 2, C and D; Table 1). The HCCs more commonly displayed a trabecular histological pattern (57/82 cases, including the clear-cell variant), but the solid (19/82 cases) or pseudoglandular (adenocarcinoma, 6/82 cases) types were also detectable (Figure 4, C–E). Sometimes foci of trabecular carcinomas originated within HCAs, producing the so-called nodule-in-nodule histotype. Each HCC histological pattern varied from well differentiated to poorly differentiated, the latter composed of anaplastic cells in intense mitotic activity, sinusoid dilatation, proliferation of neocapillaries, presence of unaccompanied arteries, and large areas of hemorrhagic necrosis. In addition, peritumorous tissues also contained clusters of small dysplastic hepatocytes characterized by reduced cytoplasm and a higher nuclear/cytoplasmic ratio, resulting in nuclear crowding and increased cell density (Figure 5A). These clusters of small dysplastic cells, in contrast to the enlarged hepatocytes, were mostly observed around portal spaces. In the late stages of neoplastic development, we also consistently noticed in the tumors proliferation of small ductular-like cells resembling oval cells, arranged in clusters together with inflammatory cells or scattered among tumor cells and frequently organized in a duct-like pattern. Clusters of oval-like cells were also present sometimes at the border between small dysplastic cells and tumor cells (Figure 5B), whereas the nontumorous parenchyma contained variable amounts of scattered oval-like cells surrounding the original giant dysplastic hepatocytes and forming numerous ductular structures (Figure 5C). In contrast to dysplastic hepatocytes, all of these small ductular-like cells were stained positive with the A6 monoclonal Ab specific for mouse bile epithelial cells and oval cells.[23] Furthermore, approximately 50% of the tumors contained large areas positive for the same staining (Figure 5D).

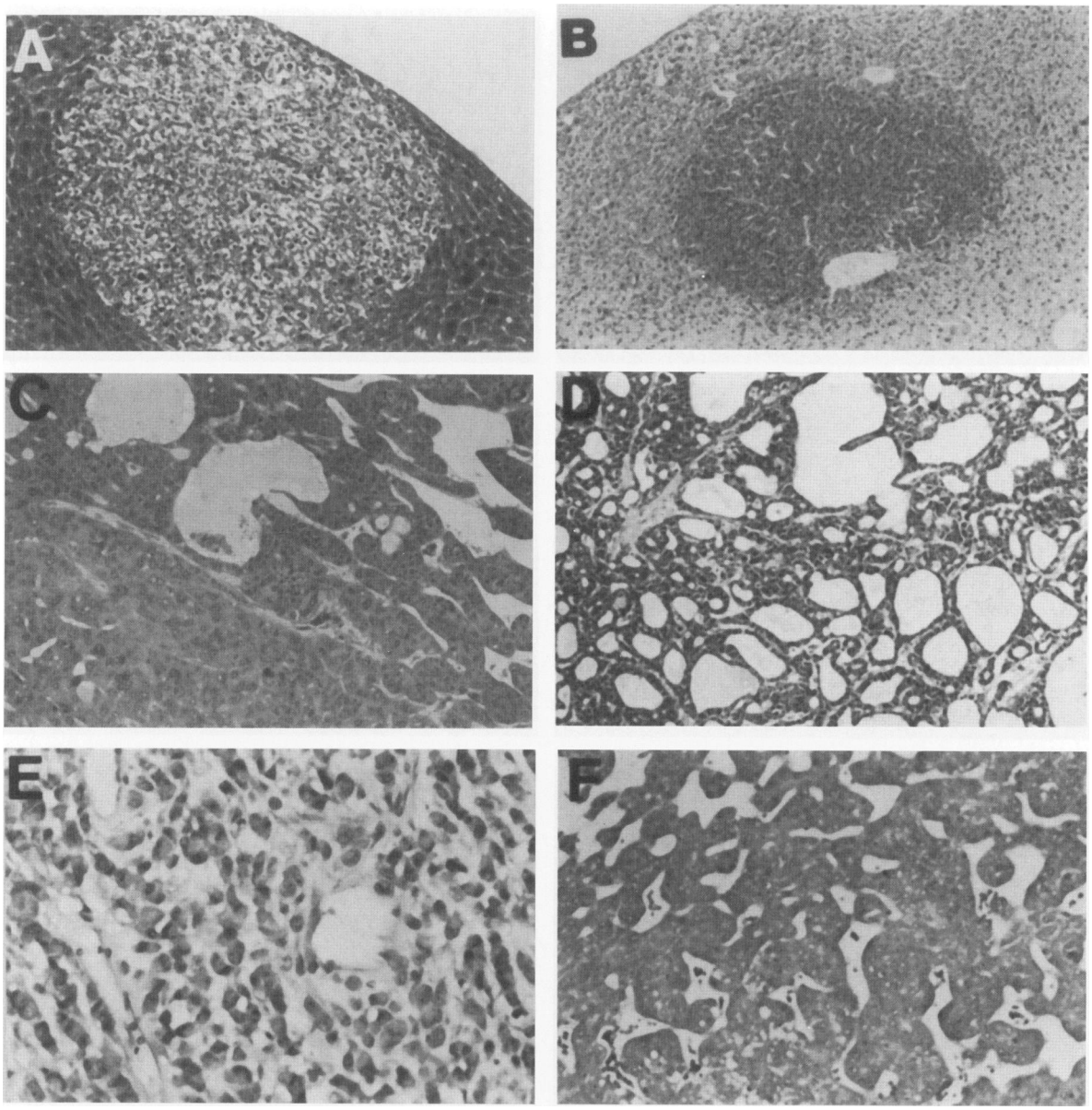

Figure 4. *Examples of preneoplastic and neoplastic lesions in c-*myc*/TGF-α* (A *to* E) *and c-*myc (F) *transgenic mouse liver.* A: *Clear-cell focus (6-month-old female).* B: *Basophilic focus (3-month-old male).* C: *Trabecular HCC (8-month-old male).* D: *Pseudoglandular HCC (5-month-old male).* E: *Poorly differentiated HCC (9-month-old female).* F: *Trabecular HCC (16-month-old male). H&E, magnification, ×100* (A *and* D); *×80* (B); *×150* (C *and* F); *×300* (E).

AFP mRNA was expressed in the carcinomas, but it was not detected in the dysplastic lesions (Figure 6, A and B). The glucose-6-phosphatase activity was strongly positive in the normal-looking hepatocytes, but the histochemical staining was weaker or negative in the dysplastic cells, foci, and tumors (data not shown). The dysplastic cells and clear-cell foci were strongly PAS positive, similar to normal hepatocytes, whereas the vast majority of tumors, the oval-type cells, and the proliferating ductular cells were PAS negative (Figure 6, C and D).

In every age group, the morphological changes were more pronounced and appeared more rapidly among male c-*myc*/TGF-α mice than those seen in female mice (Table 1). Only dysplasia developed at the same time in animals of both sexes, but the males had a more frequent and severe one as well as a higher frequency of preneoplastic and neoplastic lesions. Moreover, the tumor size was bigger in male than in female double-transgenic mice (average size of HCCs in males was $1.9 \times 1.9 \pm 0.4 \times 0.7$ cm; average size of HCCs in females was $0.6 \times$

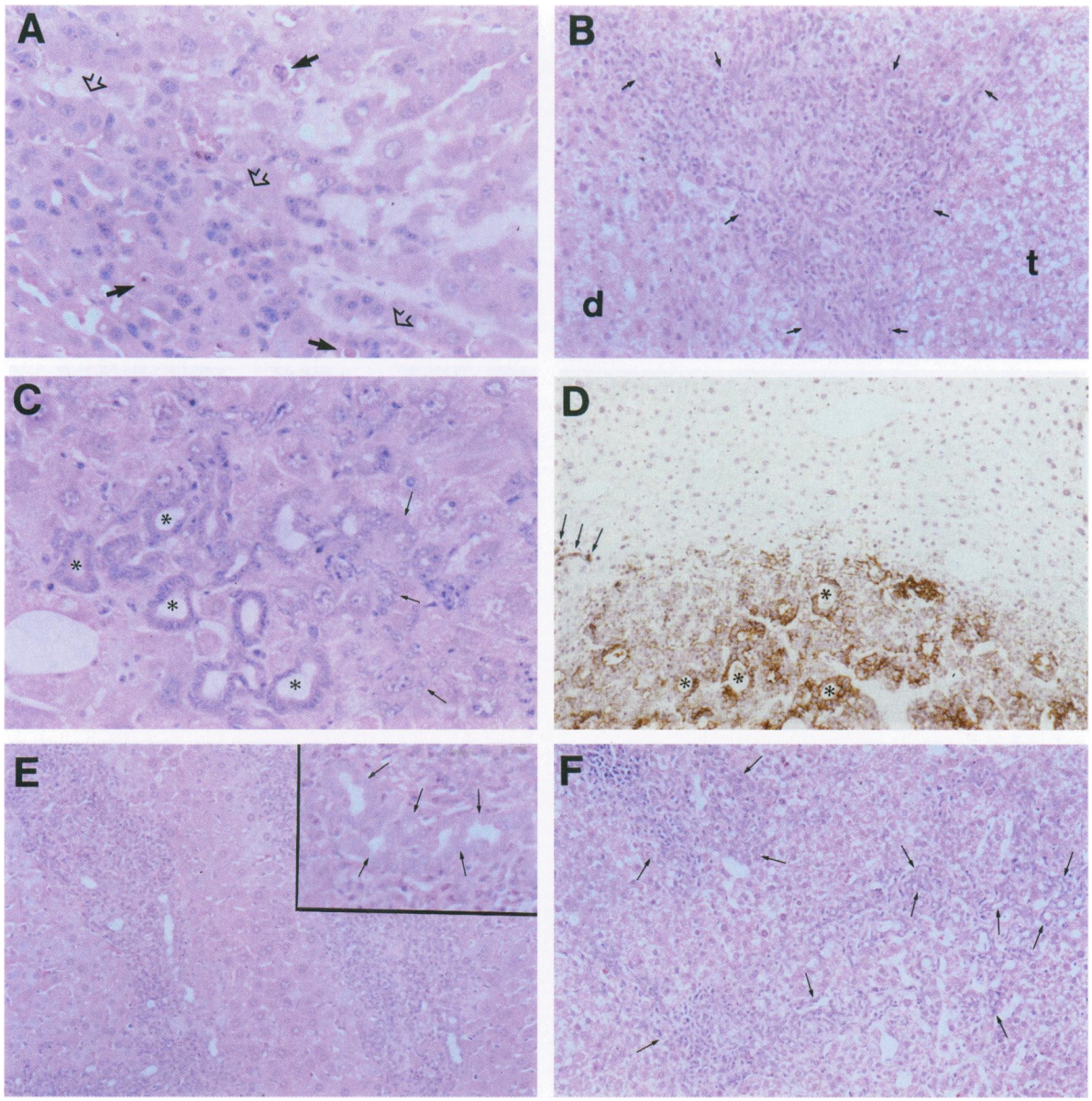

Figure 5. *Types of cell populations in c-myc/TGF-α (A to D) and c-myc (E and F) peritumorous and tumorous tissues.* A: *Cluster of small dysplastic hepatocytes* (open arrows) *in the tissue surrounding a tumor of an 8-month-old male mouse. These cells have little cytoplasm, resulting in increased cellular and nuclear density compared with larger dysplastic hepatocytes* (upper right). *Both populations show apoptotic cells* (thick arrows). *H&E; magnification, ×300.* B: *Accumulation of oval-like cells and inflammatory cells* (thin arrows) *at the border between tumor cells (t) and dysplastic hepatocytes (d) mostly of the small type (10-month-old female). H&E; ×150* C: *Peritumorous parenchyma in a 7-month-old male mouse. Oval-like cells* (thin arrows) *penetrate into the hepatic lobulus, coming in close contact and surrounding pre-existing giant dysplastic hepatocytes as well as forming numerous ductular structures* (asterisks). *H&E; ×250.* D: *A well differentiated trabecular HCC is specifically stained with monoclonal Ab A6* (lower part), *whereas in the dysplastic peritumorous tissue* (upper part) *only ductular cells are positive* (thin arrows). *Notice that many strongly immunoreactive neoplastic cells are organized in a duct-like pattern* (asterisks) *(10-month-old female). Indirect immunoperoxidase with A6 MAb counterstained with hematoxylin; ×100.* E: *Large clusters of oval-like cells and inflammatory cells around portal areas in peritumorous tissue of a 16-month-old male mouse. Oval-like cells are often arranged in ductular structures* (inset, thin arrows). *H&E; ×100 and ×250* (inset). F: *Trabecular HCC containing oval-like cells* (thin arrows) *arranged in clusters with inflammatory cells or scattered and forming ductular structures among tumor cells. H&E; ×125.*

1.1 ± 0.3 × 0.5 cm), and the neoplastic lesions in the male mice were frequently multiple and confluent, replacing almost the entire liver parenchyma.

The first perivascular dysplastic hepatocytes were detected in the c-*myc* monotransgenic mice at the age of 4 months, but severe and widespread dysplasia only appeared in males older than 1 year (Figure 2A and Table 1). At this time, most of the animals had preneoplastic foci and HCAs, and more than 30% were affected by HCCs (Figure 2, B–D). At 20 months of age, the incidence of HCCs in the male monotransgenic c-*myc* mice was approximately 67%

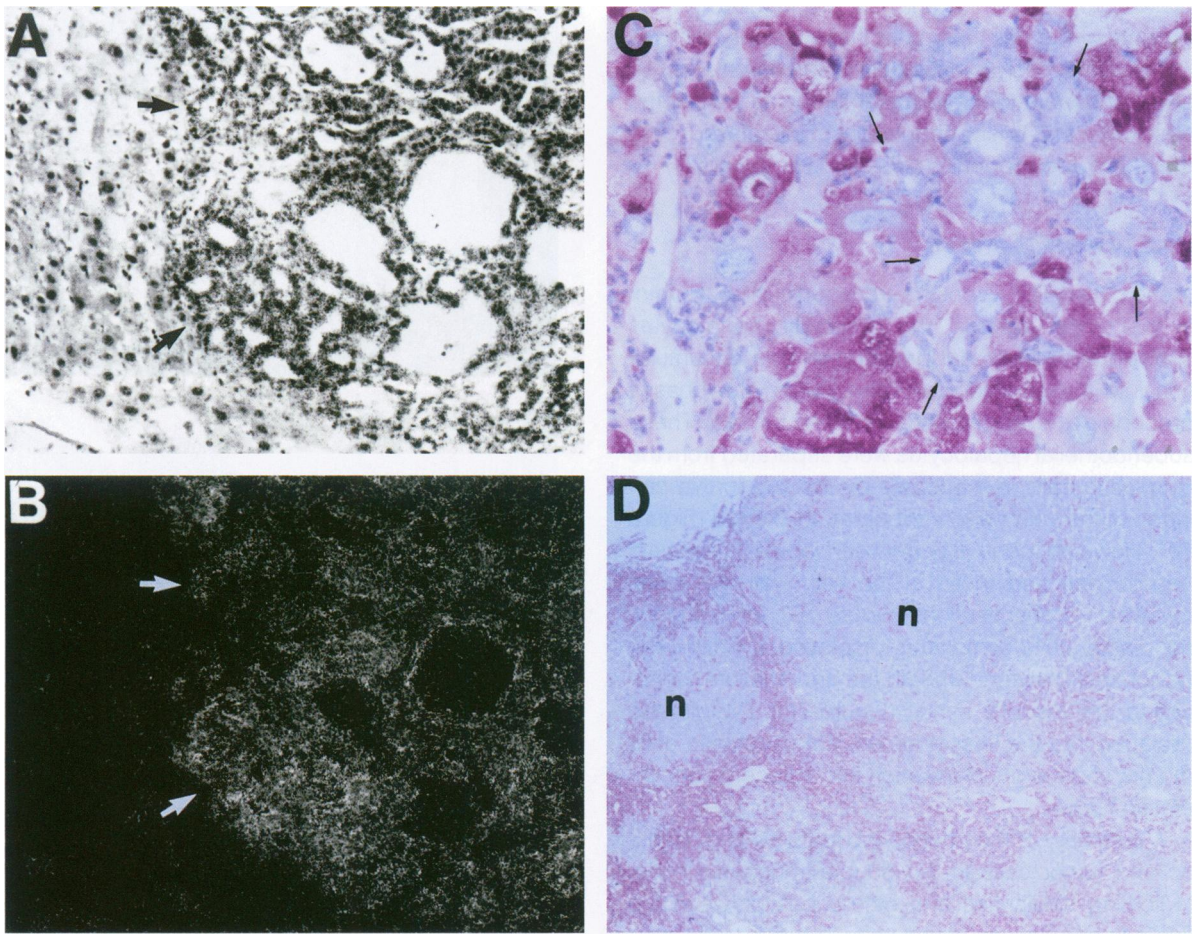

Figure 6. *Different phenotypic features of neoplastic and dysplastic tissues in c-myc/TGF-α mice.* A *and* B: *Bright- and dark-field illumination, respectively, of ISH with AFP riboprobe, in a transgenic liver harboring pseudoglandular HCC* (thick arrows). *The dysplastic peritumorous tissue does not express AFP mRNA. Magnification, ×80.* C *and* D: *PAS reaction for glycogen storage on paraffin sections, showing increased glycogen content in dysplastic hepatocytes* (C). *Oval-type and ductular cells* (arrows) *and neoplastic nodules* (*n*) (D) *are PAS negative. Magnification, ×300* (C) *and ×80* (D).

(Figure 2D and Table 1B). The tumors in the c-*myc* females, as in the double-transgenic mice, appeared later and with lower frequency than in the males (Table 1B), indicating that sex hormones may play an important role in promoting c-*myc*-induced carcinogenesis. The neoplastic lesions of c-*myc* single-transgenic mice resembled those present in the double-transgenic c-*myc*/TGF-α mice (Figure 4F), but adenocarcinomas were not observed. Of 58 animals with HCCs, 42 had well differentiated trabecular carcinomas, 9 had moderately differentiated solid carcinomas, and 7 had both types. The average size of c-*myc* HCCs was smaller than in the c-*myc*/TGF-α mice (1 × 1.4 ± 0.2 × 0.3 cm in c-*myc* male animals). Similar to the double-transgenic mice, the peritumorous and tumorous tissues of c-*myc* single-transgenic mice displayed proliferation of oval-like cells, predominantly as clusters forming ductular structures (Figure 5, E and F).

To date, two c-*myc*/TGF-α transgenic mice showed metastasis in the lungs and one c-*myc* mouse had metastasis in the lungs and the spleen. Deaths of transgenic mice were mostly caused by enormous liver masses compressing neighboring organs and by massive hemorrhages in the abdominal cavity. We did not observe any of the lesions described above in wild-type mice of each sex sacrificed between 1 and 20 months of age with similar (C57BL/6JxCBA/J)×CD1 genetic background.

Additional Analysis of Dysplastic and Neoplastic Lesions

Due to the early and consistent appearance of dysplastic hepatocytes in the c-*myc*/TGF-α transgenic mice, we undertook further investigation of the dysplastic lesions during the initial stages of the neo-

plastic process. In this analysis, we focused on two aspects of the cell biology of these lesions, namely, the capacity of dysplastic cells to replicate and apparently penetrate the vascular lumen as well as the possibility that a subpopulation of dysplastic cells might provide progenitors for the liver tumors.

Vascular Involvement

In the c-*myc*/TGF-α double-transgenic mice, but not in the c-*myc* animals, the dysplastic hepatocytes showed subendothelial proliferation and accumulation into the centrilobular veins during the phases preceding the appearance of frank tumors. These subendothelial hepatocytes were separated from the dysplastic cells in the lobule by the supportive collagen of the vein, which appeared thickened (Figure 7A). However, continuity between lobular dysplastic hepatocytes and those in the vein wall was often revealed in serial sections (Figure 7B). Interestingly, neoplastic cells sometimes displayed a similar behavior and grew underneath the endothelium, penetrating the vascular lumen (Figure 7C). Despite the prolapse into the vessel lumen, the dysplastic cells did not show signs of invasion into adjacent nondysplastic tissue.

A summary of the phenotypic features of dysplastic and tumor cells in the double-transgenic mice, indicating similarities and differences between the two cell populations is shown in Table 2.

Patterns of Gene Expression

All of the double-transgenic mice analyzed expressed the human TGF-α transgene as assessed by Northern blot analysis and immunohistochemistry, but the pattern of expression varied according to the lesions examined. We had earlier shown that the TGF-α transgene is preferentially expressed in the perivascular dysplastic hepatocytes in these double-transgenic mice.[8] Therefore, it was not surprising to find that the dysplastic cells showing subendothelial proliferation and apparently invading the hepatic veins expressed particularly high levels of TGF-α (Figure 8, A and B). Similar preferential expression of the c-*myc* transgene was not detected (data not shown). Also, before the appearance of neoplastic lesions, PCNA staining revealed the highest labeling index among the pericentral dysplastic cells, indicating intense DNA synthesis (Figure 8C) and consistent with the frequent presence of mitotic figures in these areas. In general, the dysplastic hepatocytes showed an even TGF-α staining of their cytoplasm and/or their membrane and little variability among

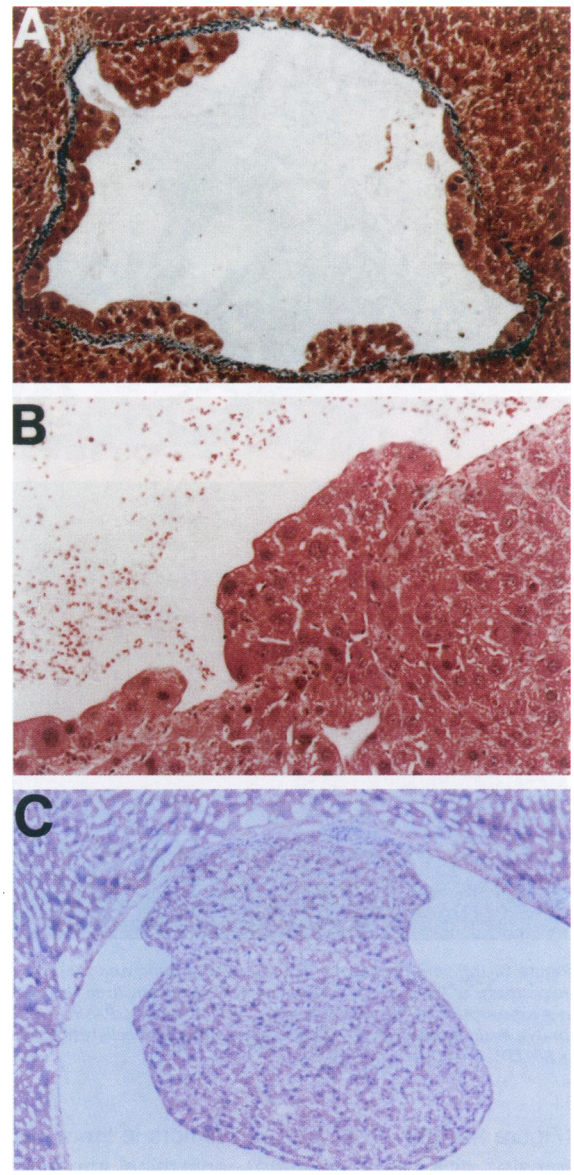

Figure 7. *Blood vessel invasion by dysplastic* (**A** *and* **B**) *and neoplastic* (**C**) *cells in double-transgenic mice.* **A**: *Masson's trichrome staining showing subendothelial proliferation and accumulation of dysplastic hepatocytes into a centrilobular vein* (*2-month-old male*). *Magnification,* ×*150.* **B**: *High power view of the invasive cells showing continuity between the dysplastic cells on both sides of the blood vessel wall* (*3-month-old male*). *H&E;* ×*300.* **C**: *Neoplastic cells penetrating the vascular lumen* (*6-month-old male*). ×*300.*

positive cells of the same area. The majority of the cells in clear-cell foci, in contrast to the dysplastic cells and basophilic foci, expressed a low level of TGF-α, but there were always a few cells that displayed very strong TGF-α expression (Figure 8B). The intensity and pattern of staining in the tumors was more variable than in the nontumorous tissues. HCCs exhibited stronger TGF-α positivity than that in the surrounding tissues and HCAs (Figure 8D), but the type of staining varied from different cytoplasmic

Table 2. *Phenotypic Features of Dysplastic Cells and Tumors in c-myc/TGF-α Transgenic Mice*

Phenotype	Dysplastic cells*	Tumors
TGF-α transgene†	+	++
Endogenous TGF-α	+	++
c-*myc*-transgene‡	+	+/++
Endogenous c-*myc*	NC/+	++
EGF-R§	NC/−	NC/−/(+)
PCNA	+	++
TGF-β1	++	+
uPA	+	++
G6P	−	−
PAS	++	−
AFP	−	+
A6	−	+

Results are presented as the comparison to the liver of wild-type mice: NC, no change; +, increase; ++, strong increase; −, reduction. See text for methods used to assess the expressions of these phenotypes.

*Include dysplastic changes in nontumorous livers and in peritumorous tissues.

†Tumors of the clear-cell variant had scattered strong TGF-α expression. Trabecular, solid, and pseudoglandular HCC had homogeneous strong expression.

‡The expression of the c-*myc* transgene in tumors was variable compared with that in dysplastic cells.

§EGF-R expression in tumors was either unchanged, diminished or, less frequently, increased (in parentheses) compared with wild-type mice and dysplastic cells.

patterns (diffuse, paranuclear, or punctate inclusion type) to a membranous type or in most of the cases a mixed type (Figure 8D), resembling patterns recently described in human HCCs.[27,28] PCNA staining demonstrated a good correlation with TGF-α expression and labeling index in the neoplastic lesions (data not shown).

Northern blot and phosphorimager analyses of the TGF-α transgene in c-*myc*/TGF-α mice, in apparent similarity with the findings reported by Takagi et al[24] in TGF-α transgenic mice treated with chemical carcinogens, showed comparable high levels of mRNA in tumors and adjacent nontumorous liver tissues (Figure 9A). On the same blots, we were also able to reveal the induction of the endogenous TGF-α mRNA in the double-transgenic mice, which reached more than fourfold in the tumors. Taken together, our results with immunohistochemistry and Northern analysis suggest that post-transcriptional mechanisms as well as induction of the endogenous growth factor may account for the higher TGF-α expression in the c-*myc*/TGF-α tumors relative to adjacent tissues.

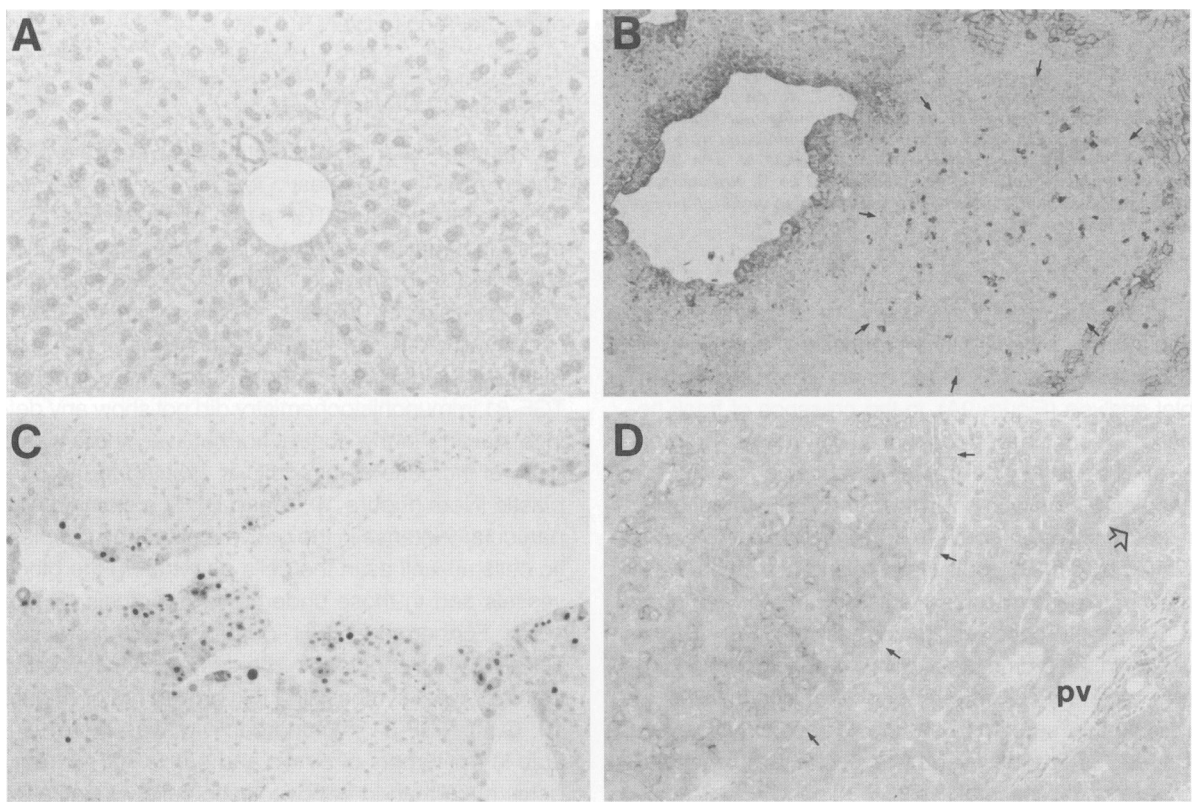

Figure 8. *TGF-α immunostaining in normal mouse liver* (A) *and* c-myc/*TGF-α transgenic liver* (B *and* D). B: *TGF-α is highly expressed in perivascular dysplastic hepatocytes, particularly those invading the central veins. In a clear-cell focus* (arrows) *a few cells show very strong immunoreactivity* (*3-month-old male*). C: *Preferential expression in perivascular dysplastic hepatocytes correlates well with their positivity for PCNA immunostaining* (*2-month-old male*). D: *HCC* (arrows) *showing stronger immunoreactivity than surrounding tissue composed of small dysplastic cells near a portal vein* (*pv*) *and few giant hepatocytes* (open arrow). *Magnification,* ×*125* (A); ×*80* (B); ×*100* (C *and* D).

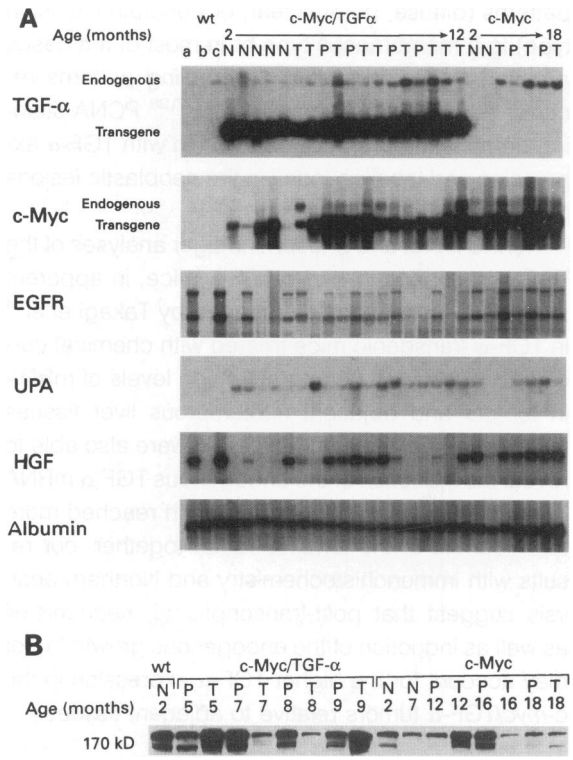

Figure 9. A: *Representative Northern blot analysis of temporal gene expression in normal wild-type (wt), c-myc/TGF-α and c-myc livers.* Lanes a *and* b, *2-month-old and 16-month-old wild-type liver;* lane c, *3-month-old wild-type liver 72 hours after partial hepatectomy as a positive control for expression of endogenous genes during liver growth. N, nontumorous; P, peritumorous; T, tumorous transgenic livers. The sizes of the mRNAs are as follows: endogenous and transgenic TGF-α, 4.5 and 1.4 kb, respectively; endogenous and transgenic c-myc, 2.3 and 1.9 kb, respectively; EGF-R, 10.5 and 6 kb; uPA, 2.4 kb; hepatocyte growth factor, 5.9 kb; albumin, 2.2 kb.* B: *Representative Western blot analysis of the EGF-R expression in livers of normal wild-type and transgenic mice.*

In the c-*myc* single-transgenic mice during the period of time preceding tumor formation, the staining for TGF-α was very weak. However, as shown by Northern blot analysis (Figure 9A), in the presence of frank hepatic tumors, the endogenous TGF-α was progressively induced up to the levels observed during the neoplastic development in the c-*myc*/TGF-α transgenic mice, resulting in some TGF-α immunoreactivity displayed by c-*myc* neoplastic cells and to a much lesser extent by peritumorous dysplastic hepatocytes (data not shown). These results suggest that TGF-α is involved in the promotion of c-*myc*-induced carcinogenesis, and conceivably, one of the mechanisms by which c-*myc* supports tumorigenic transformation via a TGF-α/EGF-R autocrine growth mechanism[18] may simply be the induction of the TGF-α ligand.

Northern blot analysis employing the mouse c-*myc* cDNA transgene probe showed that the vast majority of mice in both transgenic lines expressed high levels of the c-*myc* transgene, with variations between tumors and surrounding tissue (Figure 9A). Moreover, we found an increase of the endogenous c-*myc* in most neoplastic lesions of single- and double-transgenic mice and to a lesser extent in nontumorous tissues (Figure 9A). Taken together, our results on the expression of transgenes and their endogenous counterparts are in apparent conflict with those reported by Sandgren et al[9] showing in c-*myc*/TGF-α mice enhanced expressions of both transgenes in hepatic tumors relative to those in adjacent liver, without the induction of endogenous genes.

We also examined the levels of EGF-R to elucidate whether the growth-promoting signals depended on the EGF-R status and whether c-*myc* could sensitize transgenic hepatocytes to TGF-α not only by inducing the ligand but also by inducing its receptor. Similar to results obtained in untreated[29] or carcinogen-treated TGF-α single-transgenic mice,[24] and by Sandgren et al[9] in the double-transgenic mice, we found high variability of the EGF-R transcript and protein in different transgenic and wild-type mice (Figure 9, A and B). We observed either no induction or a reduction or, more rarely, some increase of the EGF-R expression in the tumors from both transgenic lines as compared with adjacent nontumorous liver or normal mouse liver.

Expression of TGF-β1

As mentioned earlier, apoptotic cells were frequently observed in the dysplastic lesions. As it is well established that mature TGF-β1 is able to induce apoptosis in hepatocytes, particularly those having been subjected to promoting agents,[30–32] we examined the expression of TGF-β1 and uPA, which are known to be involved in the activation of latent TGF-β1 to its mature biologically active form.[33,34] Although TGF-β1 immunohistochemistry did not show any definite staining in the control normal liver, there was a general increase of intracellular staining in the dysplastic livers (Figure 10, A and B). The staining was particularly intense in the perivascular large dysplastic cells as well as in the cells penetrating the blood vessels and in those undergoing apoptosis (Figure 10B). Furthermore, ISH demonstrated increased TGF-β1 mRNA content in the same cells, indicating that they were synthesizing this growth factor (Figure 10, C–F). TGF-β1 immunoreactivity was more variable in the tumors of c-*myc* and c-*myc*/TGF-α transgenic mice than in the dysplastic cells. Quantitative analysis by phosphorimager scanning of Northern blots further supported an increased steady state of TGF-β1 mRNA levels in the livers of transgenic mice (data not shown).

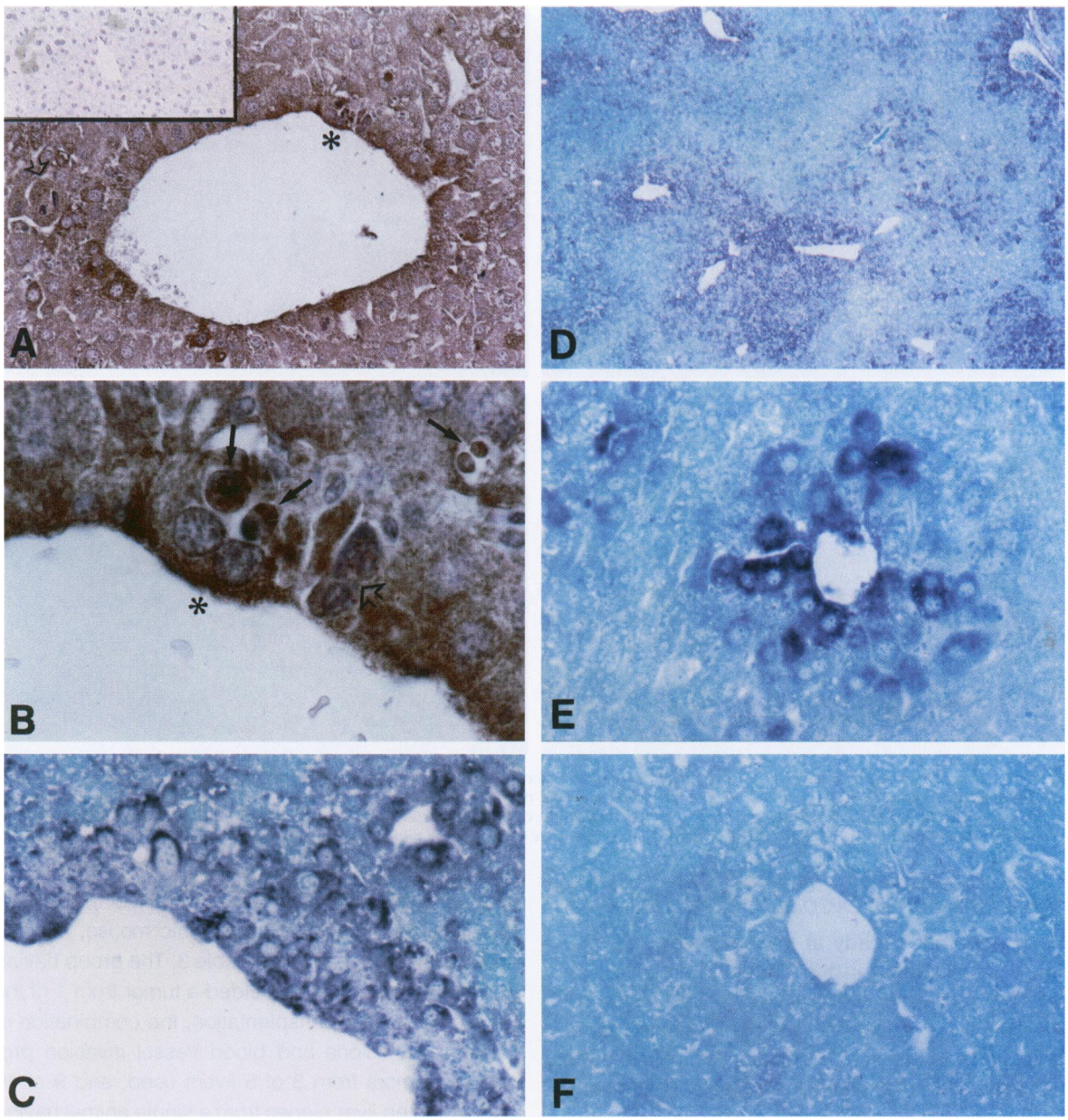

Figure 10. *TGF-β1 expression in c-myc/TGF-α transgenic liver.* **A:** *Immunostaining with Ab against mature TGF-β1 showing strong expression around a central vein compared with the liver of wild-type mice* (**inset**). *Magnification, ×125 and ×100* (**inset**). *The area indicated by an* **asterisk** *is magnified in* **B** *and shows dysplastic hepatocytes strongly positive for TGF-β1 undergoing apoptosis* (**open arrow**) *and containing apoptotic bodies* (**thick arrows**) (*3-month-old male*). *Magnification, ×1000.* **C:** *ISH with a digoxigenin-labeled TGF-β1 mRNA probe in the same area as in* **B**. *Positive reaction is indicated by dark color. Counterstained with methyl green; ×400.* **D** *and* **E:** *ISH showing strong perivascular production of TGF-β1 mRNA in dysplastic liver. ×40* (**D**) *and ×400* (**E**). **F:** *Serial section of same field as in E with sense probe. ×400.*

Immunostaining for uPA showed in the dysplastic lesions a pattern similar to that for TGF-β1. The large dysplastic and especially the invading cells were strongly positive for uPA (Figure 11, A and B). The tumors as well as the oval-like cells and the proliferating ductular structures contained in the tumors and contiguous tissues were also strongly immunoreactive (Figure 11, C and D). Northern blot analysis also showed uPA mRNA induction in the dysplastic livers as well as in the tumors (Figure 9A).

Northern blot analysis of hepatocyte growth factor (Figure 9A) and c-*met* transcripts (data not shown) did not show increased expression of this growth factor receptor system in the dysplastic livers as compared with young normal mice. The expression in tumors was variable but in general lower than in the surrounding tissues.

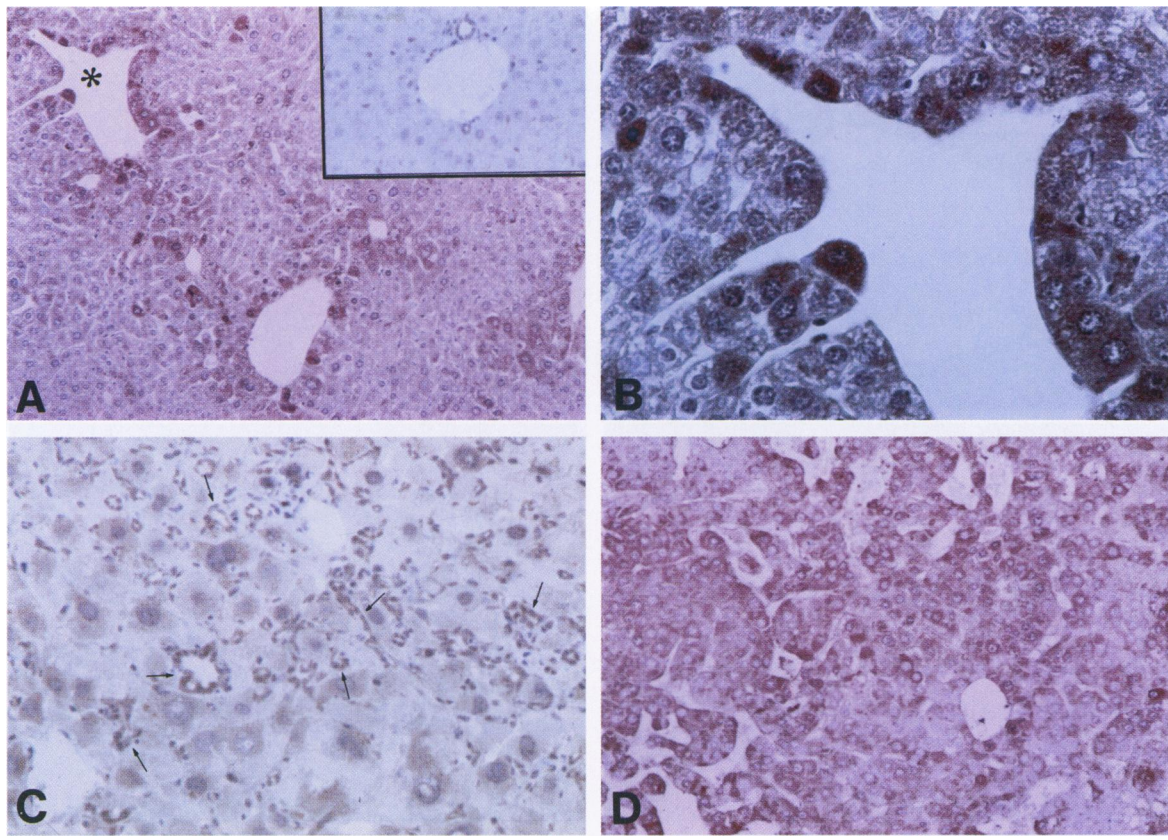

Figure 11. *Immunostaining with uPA Ab in c-myc/TGF-α transgenic mice.* A: *Dysplastic liver strongly reactive, particularly in invading dysplastic cells* (asterisk, *enlarged in* B; *magnification, × 1000*) *compared with wild-type liver in which only bile epithelial cells are stained* (inset) (*3-month-old male. Magnification, × 100.* C: *Peritumorous tissue containing oval-like cells and ductular structures* (thin arrows) *more uPA positive than giant hepatocytes* (*8-month-old male*). *× 200.* E: *HCC strongly immunoreactive* (*6-month-old male*). *× 200.*

Transplantation of Dysplastic Liver Tissue

It was apparent already at the early stage of neoplastic development in the double-transgenic liver that the dysplastic cell population was extremely heterogeneous. Therefore, we performed subcutaneous transplantation of dysplastic tissues into nude mice in an attempt to clarify the biological nature and test the oncogenic potential of dysplastic cells. The transplanted liver tissues were selected from c-*myc*/TGF-α transgenic mice having three types of lesions: 1) dysplasia without any other lesion, 2) dysplasia and apparent blood vessel invasion, and 3) dysplasia with HCC. From 6 to 12 pieces of liver tissue were transplanted from each transgenic mouse, and the results are summarized in Table 3. The group having only dysplastic lesions yielded a tumor from 1 of the 4 livers used for transplantation; the combination of dysplastic lesions and blood vessel invasion produced tumors from 3 of 5 livers used, and 9 of 10 transplanted liver pieces from a single animal having both dysplasia and carcinoma gave rise to tumors. Histological examination of the subcutaneously growing tumors showed the picture of typical HCC in every case (Figure 12).

Table 3. *Transplantation of Dysplastic Liver Tissue into Nude Mice*

Histological diagnosis in donors	Number of donors*	Age of donors (months)	Number of recipients with tumors	Number of tumors in recipient/number of transplanted pieces
Dysplasia without vascular invasion	4	1.5	1/4 (25%)	2/12 (17%)
Dysplasia with vascular invasion	5	2.5	3/5 (60%)	5/12 (42%) 2/6 (33%) 6/8 (75%)
Dysplasia with carcinoma	1	5.0	1/1 (100%)	9/10 (90%)

*Six to twelve pieces from each transgenic liver were transplanted.

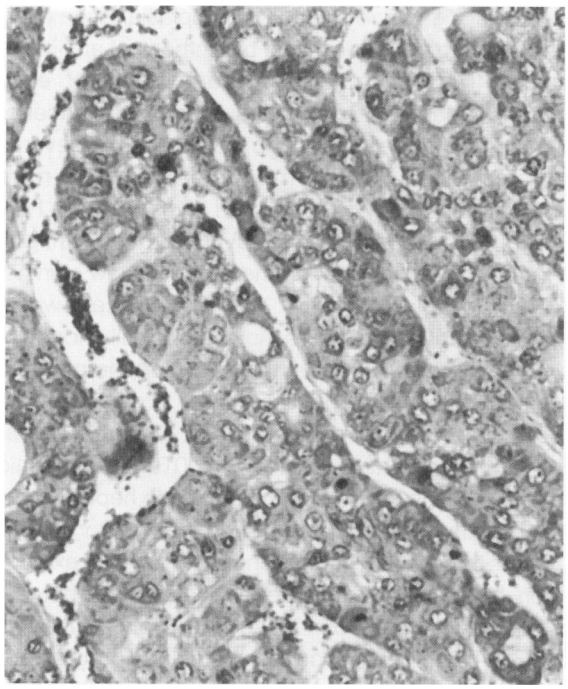

Figure 12. *Typical trabecular HCC grown on a nude mouse after transplantation of a dysplastic liver sample. H&E; magnification, ×400.*

AFP, similar to most of the liver carcinomas formed in the c-*myc*/TGF-α transgenic mice, was expressed in all of the subcutaneous tumors (data not shown), although no AFP mRNA could be found in the dysplastic livers including the cases that were transplanted.

Discussion

In the present report we describe in detail the sequential cell alterations occurring in the liver of c-*myc*/TGF-α and c-*myc* transgenic mice during neoplastic development. Both c-*myc*/TGF-α and c-*myc* transgenic mice display a stepwise pathological sequence in the liver resembling the hepatic changes previously reported in TGF-α mice.[14] However, the dramatic acceleration, extent, and severity of the hepatic lesions in c-*myc*/TGF-α mice clearly differentiate this model from either of the single-transgenic mice and show the synergistic effects of this transgenic combination. The progression from early liver dysplastic lesions and preneoplastic focal alterations to widespread dysplasia and tumors occurred within 4 months in the double-transgenic male animals, resulting in striking liver enlargement, 100% incidence of HCCs by 8 months, invasion of the abdominal cavity, and survival ≤ 1 year. In contrast, either of the transgenes alone induces less dramatic liver enlargement and remarkably longer latency as well as progression of neoplastic development, resulting in a much lower tumor incidence.

Although the tumors appeared later and with lower incidence in c-*myc*/TGF-α and c-*myc* females, it is worth emphasizing that the sequence of events, including the formation of malignant tumors, was essentially the same as that seen in the male mice. Takagi et al[29] have shown that female sex hormones exert strong inhibition on the neoplastic process in the TGF-α transgenic mice, resulting in approximately a 10-fold reduction of tumor incidence and absence of malignant tumors in female *versus* male animals. Taken together, these findings suggest that sex hormones have more control on the TGF-α-induced neoplastic events in the liver than on those caused by c-*myc*.

In the present study we observed induction of the endogenous c-*myc* and TGF-α genes in both c-*myc* and c-*myc*/TGF-α transgenic mice, whereas Takagi et al[29] previously reported overexpression of c-*myc* in a significant percentage of tumors arising in TGF-α single-transgenic animals. These observations underline the collaborative role of these two genes in providing a selective growth advantage to the tumor cells in the liver. Our results are in agreement with previous studies showing that overexpressions of c-*myc* and TGF-α in clonally derived rat liver epithelial cells cooperate in their association with tumorigenicity, possibly because c-*myc* increases the cellular sensitivity to the TGF-α/EGF-R autocrine growth signaling.[18] Also, our findings suggest that one of the mechanisms for c-*myc* to promote growth autonomy and tumorigenic transformation via this TGF-α/EGF-R autocrine growth signaling may simply be the induction of the TGF-α ligand. On the other hand, the overexpression of the endogenous c-*myc* in TGF-α and c-*myc*/TGF-α transgenic mice suggests that the growth advantage provided by these two genes may at least in part reside in an amplifying loop of mutual induction. Data from *in vitro* experiments further support our conclusions. For instance, hepatocytes overexpressing TGF-α displayed autonomous growth contributing to their tumorigenic capacity.[35] Furthermore, in transformed rat liver epithelial cells, TGF-α mRNA levels correlated with tumorigenicity only in the c-*myc*-overexpressing cells,[18] whereas activation of the endogenous c-*myc* or c-*myc* cotransfection significantly increased growth advantage and malignant transformation of rat liver epithelial cells transfected with TGF-α.[36]

The EGF-R expression was analyzed to see whether the sensitivity to proliferation signals generated by TGF-α was correlated with the levels of the

receptor. We found sometimes an increase but more frequently either no significant induction or a reduction of transcript and protein in c-*myc* and c-*myc*/TGF-α tumors as compared with nontumorous tissues and control livers. These results, along with the unchanged or decreased EGF-R mRNA levels found in tumors relative to adjacent tissues of TGF-α single-transgenic mice,[29] represent an additional phenotypic trait common to the three transgenic lines. Moreover, they suggest that the amounts of EGF-R present during neoplastic development in these transgenic mouse models were sufficient to support the TGF-α-dependent tumorigenic loop without necessarily needing further increase.

Both TGF-α and c-*myc* transgenic mice display dysplastic cells in the liver before the appearance of neoplastic lesions. It is, therefore, not surprising that the most relevant pathological change resulting from the synergistic interaction of these two transgenes is a very rapid development of severe liver cell dysplasia. This type of hepatic abnormality is widespread throughout the liver lobuli by the second month of age in the c-*myc*/TGF-α double-transgenic mice and accompanies the subsequent development of preneoplastic and neoplastic lesions.

The term liver cell dysplasia was first coined by Anthony et al[37] to define a histological abnormality of the human liver resulting in cellular and nuclear enlargement and pleomorphism as well as multinucleation of liver cells occurring in multiple groups or affecting whole cirrhotic nodules. Typical features of dysplastic cells are enlargement of both cytoplasm and nucleus, so that the nuclear/cytoplasmic ratio remains normal, intranuclear inclusions, and prominent nucleoli. Although Anthony et al[37] suggested that with time there was a progression from cirrhosis to dysplasia and ultimately to HCC, the same authors specified that liver cell dysplasia was premalignant only because it is associated with increased risk of HCC. Since then, several studies have been undertaken to delineate the role of hepatic dysplasia in human hepatocarcinogenesis but without reaching definitive conclusions.[38–42] Morphometric studies have led to the subdivision of dysplastic cells into large and small types, both possibly able to represent a tumor precursor.[43,44] However, this issue is still unresolved because the differentiation of high-grade dysplasia from neoplastic lesions is admittedly difficult and still based on arbitrary morphological criteria lacking the support of molecular genetic analyses.[45] Therefore, transgenic mouse models in which liver cell dysplasia represents one of the main phenotypic changes could be useful to gain new insights for this still unclear human liver alteration.

The appearance of hepatic dysplasia resembling the one originally described by Anthony et al[37] in the human liver is a common characteristic of early stages in hepatocarcinogenesis seen in a number of transgenic models. Although the extent and severity of the dysplastic lesions may differ, transgenic mice in which c-*myc*, TGF-α, mutated H-*ras*, HBsAg, HBx gene, and especially the SV40 large T antigen (SV40Tag) are targeted to the liver, all show signs of hepatic dysplasia,[9,11,14,46–49] the relevance of which for the neoplastic process, however, is still uncertain. In the c-*myc*/TGF-α mice, the early appearance and extensive abnormal proliferation of large dysplastic cells, followed by apoptotic death, are quite similar to those reported in the SV40Tag transgenic mice.[48,49] The c-*myc*/TGF-α dysplastic hepatocytes, particularly those located pericentrally, strongly expressed the TGF-α transgene whereas the expression of the c-*myc* transgene was present but not selectively increased in these cells. Both c-*myc* and TGF-α have been shown to stimulate progression through the G1 phase of the cell cycle, thus generating, in the case of overexpression, the conditions for uncontrolled cell proliferation (reviewed in Ref. 50). Recent findings in our laboratory indicate that, indeed, this mechanism of cell cycle deregulation by c-*myc* and TGF-α strongly contributes to the neoplastic development in the liver of double-transgenic mice (Santoni-Rugiu and Thorgeirsson, manuscript in preparation). However, uncontrolled proliferation can also be detrimental to many hepatocytes as it leads to accumulation of DNA damage and possibly exhaustion of their proliferative capacity. The presence of DNA damage has been demonstrated by the abnormal amount of chromosomal aberrations recently described in the dysplastic cells of young c-*myc*/TGF-α mice[51] and is reflected in their atypical mitotic figures. In addition, our recent data indicate that c-*myc*/TGF-α dysplastic hepatocytes are less prone to respond to partial hepatectomy than hepatocytes of c-*myc* or normal mice and also show signs of replicative senescence (Factor, Jensen, and Thorgeirsson, submitted for publication). These observations indicate that stimulation of continuous rounds of DNA replication by the transgenes in these cells may cause DNA damage and defective mitosis. In mammalian cells, wild-type p53 induction in response to DNA damage leads to growth arrest via transcriptional up-regulation of $p21^{WAF1/CIP1}$, an inhibitor of cyclin-dependent kinases that plays a key role in the growth regulation of tumor and senescent cells (reviewed in Ref. 52). Overexpression of $p21^{WAF1/CIP1}$ in certain cell lines, including hepatoblastoma HepG2 cells, induces not only growth arrest but also

loss of cytokinesis, resulting in formation of giant pleomorphic cells eventually undergoing cell death, mimicking in this way the phenomenon of cellular senescence.[53,54] We have recently demonstrated high levels of p53 and $p21^{WAF1/CIP1}$ in the dysplastic cells of c-*myc*/TGF-α mice (Santoni-Rugiu and Thorgeirsson, manuscript in preparation) and therefore believe that a possible mechanism for the genesis of large dysplastic hepatocytes may be due to a functional deficiency of hepatic DNA repair by the transgene-induced chronic proliferative stimulus followed by an inability to carry out normal cell division. This assumption is further supported by the fact that the only pathology detected in DNA repair gene (ERCC-1)-deficient mice was massive liver cell dysplasia resulting in lethal hepatic failure before weaning.[55] At this time most organs are rapidly growing, but only the liver was affected in the ERCC-1 knock-out mice that also overexpressed p53, indicating that proliferating hepatocytes are susceptible to high levels of endogenous DNA damage.

The accumulation of DNA damage, however, is not the only explanation for the phenotypic features and fate of dysplastic cells overexpressing c-*myc* and TGF-α. Dysplastic hepatocytes, particularly those undergoing apoptosis, also produced high levels of TGF-β1 and uPA. Up-regulation of TGF-β1 has been reported in human and experimentally induced HCCs.[56–59] Mature TGF-β1 is a powerful growth inhibitor for hepatocytes *in vitro* and *in vivo* (reviewed in Ref. 26) and is also capable of inducing apoptosis in these cells, particularly during liver regression.[30–32] Moreover, in these circumstances it has been suggested that hepatocytes preparing for apoptosis synthesize TGF-β1 as an induction signal.[31] There is also some experimental evidence suggesting that pericentral hepatocytes are particularly predisposed to TGF-β1-dependent apoptosis.[60] Nevertheless, the exact role of TGF-β1 in hepatocarcinogenesis is at present not well defined.[61,62] Although in most experimental systems TGF-β1 is synthesized in the nonparenchymal cells of the liver,[56,57,63,64] we recently observed, similar to our findings in the transgenic mice, TGF-β1 production in the hepatocytes of cirrhotic and tumor-containing human livers (Nagy and Thorgeirsson, unpublished observations). Comparable results in human neoplastic livers have been recently been reported by Bedossa et al.[59] In light of the compensatory restraining role proposed for TGF-β1 during liver regeneration,[26,64] it is likely that its induction in the liver of transgenic mice is an autocrine protective mechanism necessary for suppressing via cell cycle inhibition and apoptosis the abnormal cell growth stimulated by c-*myc* and TGF-α. In this manner, TGF-β1 may also contribute to the histological characteristics of large dysplastic hepatocytes.

uPA is expressed at a very low level in the normal liver,[65] but it is so frequently expressed in human HCCs that it has been proposed as a marker for HCC.[66] uPA participates in the activation of several growth factors, including TGF-β1,[33,34,67] and its production and capability of catalytically amplifying a cascade of membrane proteases have been correlated with the invasive and metastatic capacity of different tumor cells.[68,69] Increased TGF-α[70] and TGF-β1[71] production has also been associated with an invasive growth pattern of epithelial cells. Furthermore, Busso et al[72] reported recently the induction of uPA protein and its proteolytic activity in human primary hepatocytes treated with TGF-β1. Therefore, we hypothesize that an autocrine amplifying loop of reciprocal local activation between the protease and the growth factor may take place in the liver of our transgenic mice. Both dysplastic and neoplastic cells may utilize the amplifying proteolytic cascade induced by uPA to penetrate the vessels and/or expand into the liver parenchyma, although, except for the vascular prolapse, dysplastic cells showed no sign of invasion into the surrounding nondysplastic tissues. On the other hand, the activation of TGF-β1 by uPA implies that neoplastic cells possess decreased sensitivity to the cytokine to overcome its inhibitory effects (Santoni-Rugiu and Thorgeirsson, manuscript in preparation)

The subendothelial proliferation and the apparent invasive nature of the early pericentral dysplastic lesions was a prominent characteristic of the livers in the double-transgenic mice, which was not observed in the c-*myc* single-transgenic animals. Similar but less extensive vascular lesions were reported in the phenobarbital-treated TGF-α transgenic mice.[24] In those animals as well as in the c-*myc*/TGF-α transgenic mice described in the present study, no preneoplastic foci or adenomas were at early stages associated with these lesions. In contrast, invasive vascular lesions deriving from basophilic foci have been described in mice treated with diethylnitrosamine[73] and classified as microcarcinomas that are predisposed to develop into trabecular HCCs.[74] In this model of chemical hepatocarcinogenesis, the frequency of vascular invasions was positively correlated with the size of the foci and a high percentage of the invasive foci expressed AFP,[74] whereas we have not observed AFP expression in the invading dysplastic c-*myc*/TGF-α hepatocytes but only in the neoplastic cells. Due to the proteolytic activity of uPA in combination with the high expression of the

TGF-α transgene, the pericentral c-*myc*/TGF-α dysplastic hepatocytes seem capable of disrupting the vessel wall and, at least for a limited period of time, growing within the vessel lumen. However, mechanical factors, such as the absence of a centrilobular limiting plate and the openings of the sinusoids in the vein wall, might also predispose to the vascular prolapse of cells undergoing hyperplasia and/or hypertrophy. In this regard, it is important to note that in the liver of transgenic mice the capacity to invade the vascular lumen and overexpress TGF-α and uPA was also displayed by the neoplastic cells. Hyperplasia and prolapse of hepatocytes into the wall of hepatic veins have been reported in patients after long-term treatment with androgens, a possible association with increased risk of liver tumor formation.[75]

The results from the transplantation showed that the initiated cell population is already present during the early dysplastic stage of the neoplastic process and tissues harboring the combination of dysplasia and vascular prolapse yielded more tumors than those having only dysplastic lesions (Table 3). However, caution is needed in interpreting these data. All of the tumors derived from the transplanted livers were malignant and composed almost entirely of small diploid cells expressing AFP, thus, considerably different from the large dysplastic cells. Furthermore, the variation in the number of tumors yielded in recipient animals suggests a certain cellular heterogeneity within the transplanted tissues. Therefore, our data indicate that the mechanism(s) responsible for the production of dysplastic lesions in c-*myc*/TGF-α mice may either directly or indirectly generate the initiated population. However, the possibility that separate processes may generate dysplasia and neoplasia cannot be excluded. Although the nature and derivation of the initiated cell population remain to be defined, it seems unlikely that the large dysplastic hepatocytes, including those displaying vascular invasion, are the precursors. As discussed above, these cells, in large part due to the overexpression of TGF-β1, appear to be extremely vulnerable to apoptosis and account for the vast majority of apoptotic cells in the transgenic livers (Santoni-Rugiu and Thorgeirsson, manuscript in preparation). Nevertheless, the possibility that some of the dysplastic cells may be selected, for instance by becoming resistant to TGF-β, and generate tumorigenic clones after the occurrence of additional events cannot be ruled out. In this respect, it is important to note that, in both the SV40Tag[48,49] and the c-*myc*/TGF-α transgenic models, and to a lesser extent also in the c-*myc* mice, the emergence of a population of small cells occurs after the formation of large dysplastic hepatocytes. In the SV40Tag mice, these small cells were described as structurally abnormal periportal transitional cells, forming multifocal hyperplasia before the appearance of frank tumors. In the c-*myc*/TGF-α transgenic livers, small dysplastic cells constitute part of the peritumorous tissue and are mostly localized around portal areas. In the livers of 6- to 8-month-old TGF-α monotransgenic mice, Webber et al[25] described, besides large dysplastic cells with low proliferative activity, clusters of small replicating hepatocytes without, however, a preferential periportal location. These authors proposed that in this model tumors may originate from these cells. Studies in human liver have also suggested that the large dysplastic cells may be a product of an abnormal or altered liver regeneration, and the small dysplastic cell population, with a high nuclear/cytoplasmic ratio similar to liver cancer cells, is a more likely candidate for the pretumorous cell type.[43,44] Therefore, the dysplastic hepatocyte population may have essentially two options: either undergo apoptosis, which may be closely associated with the up-regulation of TGF-β1 and uPA, or evolve into initiated hepatocytes that provide the potential precursor population for the HCCs.

Alternatively, the initiated population could be derived from the oval-like cells we have detected in c-*myc*/TGF-α and c-*myc* peritumorous and tumorous tissues. There is evidence that oval or ductular cells are progenitors for HCCs in chemical models for rodents and in humans (reviewed in Ref. 76). Oval cells have been described by Sandgren et al[9] in the tumors of their c-*myc*/TGF-α and SV40Tag/TGF-α transgenic mice and by Bennoun et al[77] during hepatocarcinogenesis of SV40Tag mice, although no such cells were found in TGF-α single-transgenic mice.[14] These observations together with our data suggest that nuclear oncogenes have a critical role in the induction of oval cell proliferation. On the other hand, it seems likely that the oval cell stimulation in c-*myc*/TGF-α and c-*myc* mice may be the result of the functional deficiency of large dysplastic cells undergoing cell death. Interestingly, the progression from hepatocytes to enlarged dysplastic cells undergoing apoptosis to oval cell proliferation and ultimately tumor formation has been described in mice treated with the DNA-alkylating agent Dipin.[78] Whether the oval-like cells originating in our transgenic models participate in tumor formation or are simply a regenerative response to the liver damage remains to be defined. However, the expression of a marker for oval cells, the A6 antigen, in these cells as well as in several c-*myc*/TGF-α and c-*myc* tumors

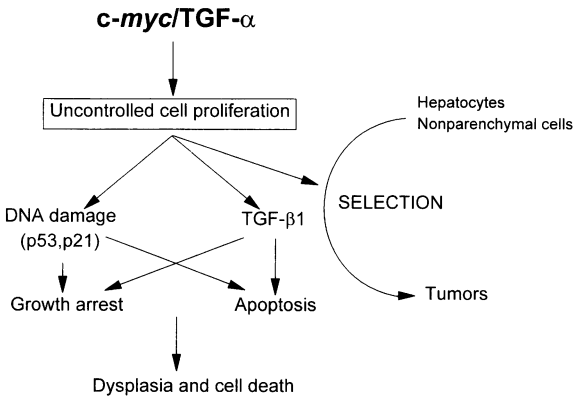

Figure 13. *Hypothetical scheme summarizing possible events caused by chronic overexpression of* c-myc *and TGF-α transgenes in the liver. Both transgenes induce continuous proliferation of hepatocytes, which produces accumulation of DNA damage resulting in generation of high-grade liver cell dysplasia and tumors. The p53-mediated response to DNA damage can lead to growth arrest and inability to perform normal cell division, resulting in formation of giant pleomorphic hepatocytes ultimately undergoing programmed cell death. The autocrine production and activation of TGF-β1 during the formation of dysplastic hepatocytes also contributes to their growth inhibition and apoptotic death. These conditions generated by overexpression of* c-myc*/TGF-α provide the selective milieu in which neoplastic cells are able to overcome both cell cycle inhibition and apoptosis.*

represents another analogy with the Dipin model, which seems to support the first hypothesis.

In summary, as indicated in Figure 13, the transgene overexpression may generate directly or indirectly heterogeneous dysplastic and nondysplastic cell populations followed by selection of neoplastic cells over the time that requires additional tumorigenic events.

Increased expression of c-*myc*[4,5] and TGF-α[27,28,79] in human HCCs has been described. TGF-α overproduction has recently been reported during chronic hepatitis B infection, and in this case, TGF-α was colocalized with HBsAg-positive cells showing signs of liver dysplasia.[27,80] The simultaneous presence of c-*myc*, TGF-α, dysplasia, and oval cells in premalignant liver diseases such as chronic hepatitis or cirrhosis, suggests that our transgenic mouse system is an appropriate model for studying the malignant evolution of these human diseases.

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