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Overexpression of Transforming Growth Factor-α Causes Liver Enlargement and Increased Hepatocyte Proliferation in Transgenic Mice

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Transforming growth factor-α (TGF-α) expression is associated with hepatocyte DNA replication both in vivo and in culture. Our previous work using TGF-α transgenic mice showed that constitutive overexpression of this growth factor in the liver causes hepatic tumors in 75 to 80% of the animals at 12 to 15 months of age. To understand the cellular events by which TGF-α overexpression leads to abnormal liver growth, we examined hepatocyte proliferative activity in young and old TGF-α transgenic mice and hepatocyte ploidy in normal, dysplastic, and neoplastic livers of these animals. At 4 weeks of age, transgenic mice had higher liver weights and liver weight/body weight ratios than non-transgenic mice of the same age and hepatocyte proliferative activity, measured by ^{3}H-thymidine incorporation after 3- and 7-day infusion, proliferating cell nuclear antigen staining, and mitotic index determination, was 2 to 3 times higher than in controls. In both transgenic and non-transgenic mice hepatocyte proliferation declined with age but the decrease was much more pronounced in control animals, so that at 8 months of age, hepatocyte replication was 8 to 10 times higher in transgenic animals. Surprisingly, however, transgenic and non-transgenic mice at this age had similar liver weight/body weight ratios. Labeling studies done in 3-month-old animals revealed that hepatocyte turnover was much faster in transgenic than in control animals, suggesting that a homeostatic compensatory mechanism involving cell death tended to restore normal liver weight/body weight ratios in older transgenic mice. Ploidy analyses showed that at 4 weeks of age transgenic mice had a higher proportion of diploid and tetraploid hepatocytes and that the hepatocellular tumors which developed in TGF-α transgenic mice at 13 months of age contained a higher fraction of diploid hepatocytes than that present in adjacent tissue or in dysplastic livers. The results demonstrate that constitutive overexpression of TGF-α causes increased hepatocyte proliferation and liver enlargement in young animals and is associated with a delay in the establishment of hepatic polyploidy. These findings as well as the response of transgenic mice to partial hepatectomy show that constitutive overexpression of TGF-α initially caused increased but regulated hepatocyte proliferation which in older animals was compensated in part by a faster cell turnover. At 8 to 10 months of age, proliferative activity may become constitutive in some TGF-α expressing hepatocytes. The data also suggest that replicative diploid and tetraploid hepatocytes, rather than large dysplastic cells are the source of the tumors which develop several months later in these animals. (Am J Pathol 1994, 145:398–408)

The expression of growth factors such as epidermal growth factor (EGF), transforming growth factor-α (TGF-α), and hepatocyte growth factor (HGF) is associated with liver regeneration induced by partial hepatectomy and chemical injury.[1,2] These three factors markedly induce DNA synthesis in hepatocytes

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maintained in primary culture in serum-free medium. The conditions which enhance or inhibit this effect as well as the interactions between these factors and other agents capable of modulating the response have been studied in detail.[3–5] However, to understand the role that EGF, TGF-α, HGF, and other growth factors may have in liver regeneration it is necessary to perform studies *in vivo*, because the sensitivity of cultured hepatocytes to these factors may differ from that of quiescent hepatocytes in the undisturbed organ. We have shown recently that while EGF, TGF-α, and HGF are active on hepatocytes in culture, their infusion into the portal vein, alone or in combination, has little effect on DNA synthesis of hepatocytes of intact rat livers.[6] We have suggested that hepatocytes placed in culture become capable of responding to these growth factors because tissue dissociation and cell isolation activate the expression of some transcription factors and proto-oncogenes.[7,8]

TGF-α expression in rat liver is developmentally regulated and increases during liver regeneration induced by partial hepatectomy or toxic injury.[5,9–13] TGF-α is a strong mitogen for hepatocytes in primary culture and is produced by hepatocytes undergoing DNA replication.[5,10] Its synthesis is also elevated in hepatocytes and transformed liver epithelial cells that are progenitors of hepatocellular tumors[14–16] and its expression increases in human hepatocellular carcinomas and in the serum of patients who have undergone partial hepatectomy.[17–19]

To examine the role of TGF-α on the growth of the liver and epithelial tissues *in vivo*, we and others have developed transgenic mouse lines which constitutively express the factor at high levels.[20] In the various strains of TGF-α transgenic mice developed so far, enlargement or altered growth of the liver, pancreas, intestine, stomach, mammary gland, and skin have been observed, depending on the promoter used to drive the transgene.[20–24] In transgenic mice of the MT42 line which carry human TGF-α cDNA driven by the metallothionein I promoter, centrilobular hypertrophy is present at 1 month of age and liver tumors develop in 75 to 80% of animals older than 12 months.[20,25,26] Other work has shown that TGF-α/c-*myc* double transgenic mice develop liver tumors by 3 to 5 months of age and that TGF-α transgenic animals are particularly sensitive to tumor promoters and initiating agents.[27–29] To understand the mechanisms by which TGF-α leads to abnormal hepatic growth in these animals, we analyzed the proliferative activity and ploidy of hepatocytes of TGF-α transgenic mice at various ages. We show here that constitutive TGF-α overexpression in the liver has marked effects on liver size, hepatocyte replication, and the establishment of polyploidy in hepatic tissue.

Materials and Methods

Animals

The construction of the TGF-α transgenic mice used in this study has been previously reported.[20,25,26] The transgenic line was originally established by injection into fertilized eggs of CD1 mice of an expression construct consisting of the zinc-inducible mouse metallothionein I promoter, a 917-bp fragment of human TGF-α cDNA (hTGF-α) and polyadenylation sequences from the human growth hormone gene.[20] Animals used in this work were from the homozygous subline 42H, derived from the MT42 line in which the transgene was stably integrated at a single site, with two copies of hTGF-α per haploid genome. The patterns of TGF-α expression in the liver of 42H and MT42 mice have been described in detail.[20,25,26] Control animals were non-transgenic CD1 mice. All animals were maintained and cared for in accordance with NIH guidelines for animal care.

Determination of Hepatocyte Proliferative Activity

Groups of TGF-α transgenic and CD1 control mice at 4 weeks or 8 months of age received ^{3}H-thymidine by infusion with Alzet osmotic pumps (Alza Corp., Palo Alto, CA) implanted subcutaneously. The mice received 1 μCi/hour of ^{3}H-thymidine for 3 or 7 days using, respectively, model 1003D (1.0 μl/hour, capacity 100 μl) and model 2001 (1.0 μl/hour, capacity 200 μl) pumps. At the end of the infusion period, the mice were killed, the livers removed, and sections prepared for autoradiography as previously described.[4,5] For determination of proliferating cell nuclear antigen (PCNA) staining, livers were fixed in methanol, embedded in paraffin, and sectioned. Monoclonal antibody to PCNA (Boehringer Mannheim Biochemicals, Indianapolis, IN) was used at 1:200 dilution and the sections were counterstained with Giemsa. The percentage of ^{3}H-labeled or PCNA-stained hepatocytes was determined after counting 1000 cells (no less than three fields per section). Hepatocyte mitotic index is expressed as the percentage of hepatocytes with mitotic figures counted on hematoxylin and eosin-stained sections (2000 cells counted). For determinations of thymidine labeling, PCNA staining and mitotic index, four transgenic and four control mice were used for each analysis.

Hepatectomies

Three-month-old CD1 and TGF-α transgenic mice were used in these studies. Partial hepatectomies were performed in mice anesthetized with methoxyflurane by separately ligating and resecting the median and left lateral lobes of the liver which constitute approximately two-thirds of the liver. The gallbladder was removed with the median lobe. All operations were performed between 9 AM and noon. Animals were killed 24, 30, 39, and 48 hours postoperatively. Three CD1 and three transgenic mice were used for each time point and were injected with 50 μCi ^{3}H-thymidine intraperitoneally 1 hour before death. Three unoperated animals (time 0) also received thymidine injection 1 hour before death. The livers were removed and frozen in liquid nitrogen. Determination of thymidine incorporation into DNA was done as previously described.[5,6]

Measurement of Hepatocyte Turnover

Three-month-old CD1 and TGF-α transgenic mice were used in these studies. Alzet osmotic pumps (model 1003D, 100 μl capacity) containing ^{3}H-thymidine (1 μCi/μl) were placed subcutaneously in these mice. Each mouse received 1 μCi of ^{3}H-thymidine per hour for 3 days and the pumps were removed 3 days after inserting them (day 0). Pairs of animals were killed on days 0, 7, 14, and 28. The livers were fixed in buffered formalin and sections prepared for autoradiography as previously described.[5,6]

Ploidy Analysis

Hepatocytes were isolated from the livers of 1-, 6-, and 13-month-old mice by a modification of Seglen's method.[30] For each experiment, three transgenic mice and three CD1 control animals were used. The livers were perfused via the inferior vena cava with Ca^{2+}, Mg^{2+}-free Hank's balanced salt solution (HBSS) followed by 0.05% collagenase/HBSS. Cells were gently dissociated from the liver capsule and washed twice before undergoing Percoll purification to yield a final suspension of viable (>90% by Trypan blue exclusion) hepatocytes. In tumor-bearing mice, this method dissociated only the nontumorous parenchyma; the tumors did not blanch during the perfusion and remained grossly intact. Tumors were subsequently isolated from the surrounding liver and tumor cells released by gentle mincing and stirring in 0.05% collagenase/HBSS at 37 C for 30 minutes.

Flow cytometry (FACS) was performed by FAST Systems, Inc. (Gaithersburg, MD) on cells which were washed and fixed in 70% ethanol and then permeabilized and stained with propidium iodide in the presence of RNAse. Analysis of DNA content was performed on a Coulter EPICS 700 scanner using standard software. Diploid mouse lymphocytes and the near-tetraploid mouse hepatocyte line AML12[31] served as reference standards for the flow cytometry and image analysis procedures. For image analysis, isolated hepatocytes were washed and resuspended in phosphate-buffered saline. Cell smears were prepared on poly-L-lysine-coated slides, fixed in 10% buffered formalin for 30 minutes, and then stained with a modified Feulgen's DNA staining kit (Cell Analysis System) and examined with a CAS 200 instrument (Becton Dickinson, Elmhurst, IL). A minimum of 200 cells were examined in each analysis.

Results

Liver Mass and Liver Weight/Body Weight Ratios in Normal and TGF-α Transgenic Mice

We wished to determine whether the constitutive overexpression of TGF-α, a strong mitogen for cultured hepatocytes, would alter the liver weight/body weight ratio in mice. This ratio is constant in normal adult rodents and humans and is rapidly restored to normal when disrupted.[1,2] The data in Table 1 show that in 4-week-old mice the liver weight/body weight ratio was approximately 60% higher in TGF-α transgenic than in control CD1 mice. Although the body weights of transgenic mice were approximately 10% lower than those of the controls, their liver weights were 46% above those of controls. Thus, these measurements indicate that TGF-α transgenic mice at 4 weeks of age have an elevated liver weight/body weight ratio which is due primarily to liver enlargement.

Table 1. *Liver and Body Weights in 4-Week-Old Normal and TGF-α Transgenic Mice*

	Body Weight (g)	Liver Weight (g)	LW/BW Ratio (×100)
Control			
1	16.5	0.76	4.6
2	20.7	1.16	5.6
3	18.0	0.90	5.0
4	18.6	1.04	5.6
Mean ± SD	18.4 ± 1.7	0.96 ± 0.17	5.2 ± 0.46%
Transgenic			
1	16.1	1.40	8.7
2	18.1	1.72	9.5
3	16.7	1.52	9.0
4	15.3	1.0	6.5
Mean ± SD	16.6 ± 1.2	1.41 ± 0.30	8.4 ± 1.3%

Hepatocyte Proliferation in Normal and Transgenic Mice

Young Animals

The increased liver mass in 4-week-old TGF-α transgenic mice could be caused by cell hypertrophy with or without changes in ploidy, increased cell proliferation, or a combination of these factors. The hepatic DNA content of the TGF-α transgenic mice was about 50% higher than that of controls (data not shown). These values are lower than those reported by Sandgren et al.[21] who found that in their line of TGF-α transgenic mice established in a different strain, the hepatic DNA content was more than twice that of controls.

We determined whether the increased liver mass and higher DNA content in TGF-α transgenic mice could be due to an elevation in hepatocyte proliferation. We used the following methods to measure hepatocyte replication in transgenic mice and CD1 controls: 1) determination of hepatocyte labeling indices after 3- and 7-day infusions of ^{3}H-thymidine by osmotic pumps; 2) PCNA staining; and 3) determination of mitotic index. As reported in the literature, hepatocyte replication is still active in normal mice at 4 weeks of age.[32] We found that after 3- and 7-day label infusion, 19 and 42% of hepatocytes, respectively, became labeled in control animals (Table 2). However, labeling of hepatocytes in TGF-α transgenic mice was double that of the controls, that is, 43 and 81% labeling indices were observed after 3 and 7 days of continuous thymidine infusion, respectively (Table 2). In other sets experiments not included here, the labeling indices after 7-day infusions were 50% and 90% in control and transgenic animals, respectively. Both PCNA staining and mitotic index determinations showed approximately twofold higher values for the transgenic animals and were consistent with the data from the labeling experiments (Table 2). These measurements indicate that the larger mass of the liver of 4-week-old transgenic mice is at least in part a consequence of increased hepatocyte proliferation.

Eight-Month-Old Animals

Using the same measurements of proliferative activity described above, we found that in control mice the hepatocyte labeling indices in 8-month-old animals were 15- to 20-fold lower than that of 4-week-old mice. Labeling indices in the older normal mice were 0.8 and 3% after 3- and 7-day ^{3}H-thymidine infusion, respectively (Table 2). In contrast, the labeling indices in 8-month-old transgenic mice after 3 and 7 days of label infusion were 6 and 25%, respectively, only four- to sevenfold lower than that of transgenic mice at 4 weeks of age. Thus, in this age group, hepatocyte proliferation in transgenic mice assessed by the labeling indices was approximately eightfold higher than that of corresponding control animals. A smaller difference between control and transgenic mice was found in PCNA staining, which was three- to fourfold higher in transgenic animals. Mitotic figures were rarely seen in normal animals, while approximately 5 figures per 1000 hepatocytes were observed in transgenic mice (Table 2).

Hepatocyte Turnover in Normal and Transgenic Mice

The results presented so far show that: 1) 4-week-old transgenic mice have increased hepatocyte proliferative activity and higher liver weight/body weight ratio; and 2) 8-month-old transgenic animals have higher levels of hepatocyte proliferation than controls. Based on these results, one would expect that older transgenic animals would also have larger livers. Instead we found that the liver weight/body weight ratios in 8-month-old transgenic mice did not differ from those of controls (5.8 ± 0.5% for controls; 5.7 ± 1.2% for transgenics). Given these results, we asked whether hepatocytes of transgenic mice may have a higher turnover than controls so that a high level of hepatocyte proliferation would be balanced by a high rate of cell death. Because we needed to obtain a sufficiently high basal labeling level so that labeled cells could be counted through an extended chase period, we per-

Table 2. *Hepatocyte Proliferative Indices in 4-Week- and 8-Month-Old Normal and TGF-α Transgenic Mice*

	4-Week-Old Mice		8-Month-Old Mice	
	Control	Transgenic	Control	Transgenic
Labeling index (3-day infusion)*	19 ± 0.9	43 ± 7	0.8 ± 0	6 ± 3
Labeling index (7-day infusion)*	42 ± 5	81 ± 5	3 ± 0.4	25 ± 3
PCNA staining†	8 ± 0.8	14 ± 3	0.8 ± 0.3	3 ± 0.7
Mitotic index‡	0.8 ± 0.2	2.1 ± 0.6	0	0.5 ± 0.4

* Expressed as percentage of hepatocytes labeled with ^{3}H-thymidine.
† Expressed as percentage of hepatocytes stained with PCNA.
‡ Expressed as percentage of mitotic figures detected in hepatocytes.

formed experiments in 3-month-old control and transgenic mice. At this age, both control and transgenic animals have ended their growth phase and the transgenic mice do not yet show the dysplastic changes present in older TGF-α transgenic animals. We infused ^{3}H-thymidine for 3 days in CD1 controls and TGF-α transgenics and killed the mice at the end of the infusion period (time 0) or at 7, 14, and 28 days later. The hepatocyte labeling indices at the end of the infusion period were 12% and 1%, respectively, for transgenic and control mice (Figure 1). While the proportion of labeled hepatocytes remained constant in normal mice through the 28-day chase period, the percentage of labeled hepatocytes decreased drastically in transgenic mice. One week after the end of label infusion, the hepatocyte labeling index in transgenic mice dropped from 12.5% to 3.2%. These data suggest that hepatocytes in 3-month-old transgenic mice have a shorter life span than in control animals. It is conceivable that the decrease in the proportion of labeled hepatocytes during the first 7 days of the chase period in transgenic mice could have resulted from dilution of the label caused by repeated cell divisions. However, this is an unlikely possibility for two reasons: 1) a large number of replicative rounds during the first 7-day period would be needed to dilute the label to values consistent with the data shown in Figure 1; and 2) the number of grains/nucleus in control and transgenic mouse hepatocytes were on average similar 7 days after the end of label infusion (data not shown).

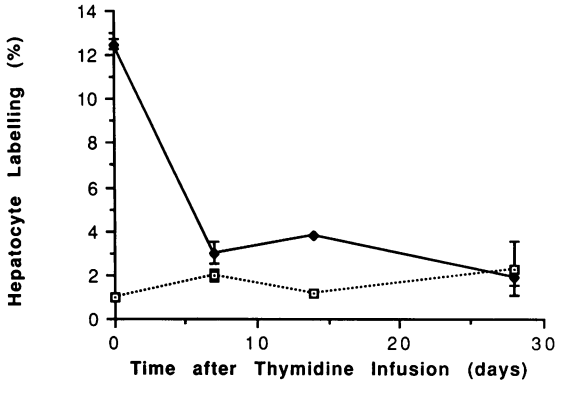

Figure 1. *Hepatocyte turnover in normal and TGF-α transgenic mice. Three-month-old normal and transgenic mice received 1 μCi of ^{3}H-thymidine per hour for 3 days infused by an osmotic pump placed subcutaneously. The pumps were removed at the end of the infusion period and two animals from each group were killed at the end of the infusion period (time 0) and 7, 14, and 28 days after. Liver tissue was fixed in formalin and sections prepared for autoradiography. The results are expressed at the percentage of labeled hepatocytes (2000 cells counted) ± SD from control (- - -) and transgenic (——) mice.*

Liver Regeneration in Normal and Transgenic Mice

The data presented above showed that hepatocytes from 3-month-old transgenic mice had a much higher proliferative index than that of the controls, but that the hepatocyte life span was considerably shorter in transgenic animals. We wished to determine how the hepatocytes from transgenic animals at this age would respond to partial hepatectomy. While one might expect that liver regeneration in TGF-α transgenic animals would result in higher levels of DNA synthesis and perhaps an acceleration of the process, it is also conceivable that no obvious differences between the two groups of animals would be found because partial hepatectomy would cause maximal replicative responses in both groups. Groups of three transgenic and three control animals were killed at 24, 30, 39, and 48 hours after two-thirds partial hepatectomy. These animals as well as sham-operated mice (time 0) received 50 μCi of ^{3}H-thymidine 1 hour before death. Figure 2 shows that, as expected, the incorporation of label into non-hepatectomized transgenic mice (time 0, sham-operated animals) was higher than in the corresponding control animals. For both groups of animals DNA synthesis reached a sharp peak at 39 hours, as described in the literature.[33,34] At this time point, the incorporation of label into DNA was twofold higher in transgenic than in control animals, while at 24 and 48 hours after partial hepatectomy DNA synthesis was three times higher in transgenic than control mice (values at 30 hours showed a smaller difference). Although it is difficult to decide from the data whether the onset of DNA synthesis after partial hepatectomy

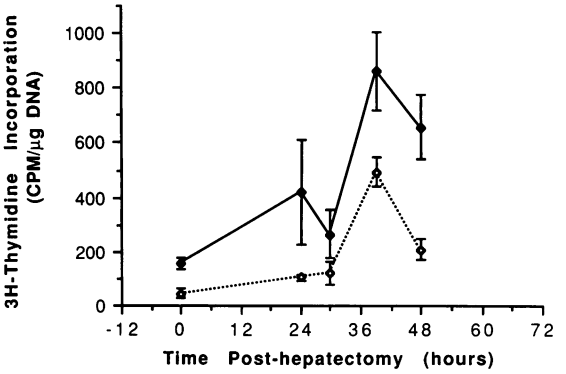

Figure 2. *Liver regeneration in normal and TGF-α transgenic mice. Three-month-old normal and transgenic mice were partially hepatectomized and three animals from each group killed at the indicated times. All mice received an intraperitoneal injection of 50 μCi of ^{3}H-thymidine 1 hour before death. The results are expressed as cpm of ^{3}H-thymidine incorporated per μg of DNA ± SD for livers of control (- - -) and transgenic (——) mice.*

occurred earlier in transgenic mice, it is clear that these mice showed a heightened proliferative response in comparison with control animals. However, because the baseline incorporation levels were higher in transgenic mice, the relative increase in DNA synthesis over the normal values measured at 39 hours was similar for transgenic and control animals.

Hepatocyte Ploidy in Young Animals

In newborn rats and mice virtually all hepatocytes are diploid. However, during the first 2 months after birth, there are drastic changes in ploidy which coincide with the almost complete cessation of hepatocyte proliferation.[35,36] In adult mice the majority of the hepatocytes are tetraploid (4N) and octoploid (8N), containing one or two nuclei, and the percentage of binucleate hepatocytes almost doubles between 3 and 6 weeks of age.[36] We determined by flow cytometry the proportion of hepatocytes belonging to various ploidy classes in 4- to 5-week-old transgenic and control animals. Figure 3 shows that at this stage, the hepatocyte population of control animals had already undergone the expected change to higher orders of ploidy. However, the change was much less pronounced in transgenic mice. In control mice there were few diploid hepatocytes (<1%), while tetraploid and octoploid cells constituted approximately 35 and 48% of the population, respectively. In contrast, in two of three transgenic animals, more than 10% of hepatocytes were diploid and the highest proportion of cells (48 to 65%) was tetraploid. The three normal animals also had a higher proportion of 8N and 16N hepatocytes than transgenic hepatocytes (with the exception of 8N ploidy level for one of the transgenic mice). Examination of the cells by image analysis did not reveal the presence of significant numbers of

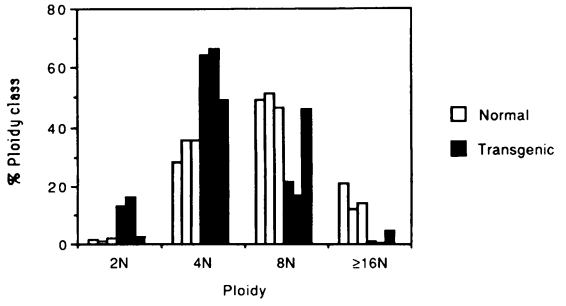

Figure 3. *Ploidy distribution of hepatocytes in young (4- to 5-week-old) normal and TGF-α transgenic mice. Hepatocytes were isolated from the livers of normal* (open bars) *and transgenic* (solid bars) *mice treated with RNAse, stained with propidium iodide, and analyzed by flow cytometry using a Coulter EPICS 700 instrument. For each set of three animals, the percentage of the cell population comprising diploid (2N), tetraploid (4N), octaploid (8N), and higher ploidy (≥16N) classes is shown.*

aggregates in cell preparations from either group, thereby excluding the possibility that cell clumping could have skewed the ploidy level distribution. We also used image analysis to determine the proportion of binucleate hepatocytes in the 4N and 8N ploidy classes. Although an equal percentage (53% and 55%, respectively) of 8N hepatocytes in both control and transgenic mice were binucleated, there was a higher proportion of 4N binucleate hepatocytes in controls (17%) than in transgenics (4%). In summary, 4- to 5-week-old transgenic mice had a higher proportion of diploid and mononucleated tetraploid hepatocytes relative to controls.

Hepatocyte Ploidy in Older Animals and in Hepatocellular Carcinomas

Large dysplastic cells were detectable by light microscopy in livers of 6- to 8-month or older transgenic mice as previously described,[25] but proliferative activity among hepatocytes was mostly confined to cells which had normal structure and size (Figure 4). Most of these cells overexpressed TGF-α as shown by experiments in which autoradiography to determine thymidine incorporation and TGF-α immunohistochemical detection were done in the same slide. In addition, in some but not all 6- to 8-month-old mice, clusters of cells which were much smaller than hepatocytes were present throughout the parenchyma, without a preferential periportal location. These cells, which may correspond to oval cells described in rat hepatocarcinogenesis and galactosamine injury,[37] were usually heavily labeled. To obtain some potential clues about the origin of the tumors which develop several months later in most transgenic animals, we compared the ploidy distribution in hepatocytes of control and transgenic mice at 6 to 8 months of age. We also determined whether hepatocytes obtained from hepatocellular carcinomas displayed different ploidy patterns from hepatocytes in older animals before tumor development. The ploidy analysis of hepatocytes from three control and three transgenic mice (Figure 5) revealed that in older animals of both groups, there was a predominance of 8N hepatocytes, while 2N hepatocytes were not detectable. In these series of experiments, the ploidy distribution of transgenic mice hepatocytes was more variable than that of the controls. However, in two of the three transgenic mice, the proportion of 16N cells was twofold higher than that of the controls (Figure 5). Analysis of the ploidy of hepatocytes from tumors which developed in three 13-month-old animals (Figure 6A) showed that 25 ± 9% of the cells were diploid and 30 ± 10% were tet-

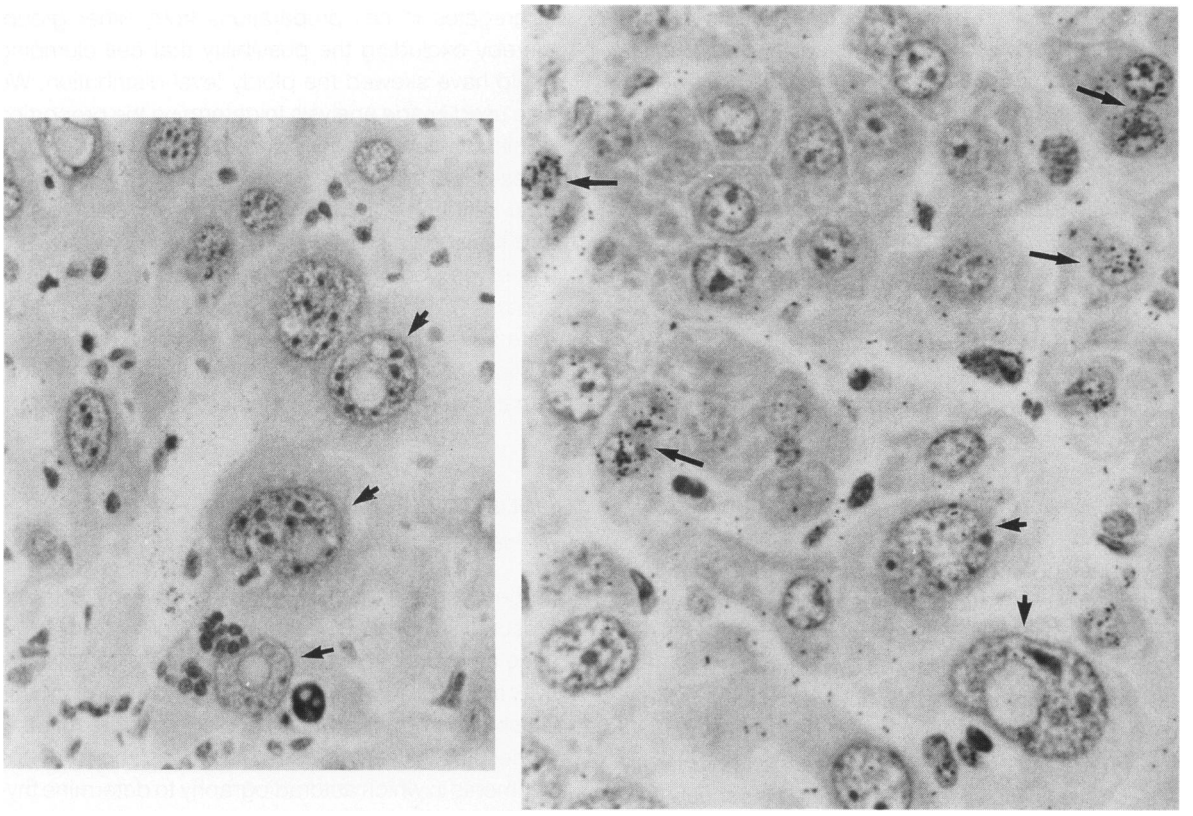

Figure 4. *Hepatocyte labeling in 6- to 8-month-old transgenic mice. Animals received a 3-day infusion of* ^{3}H-*thymidine delivered by osmotic pumps implanted subcutaneously.* **Left:** *Unlabeled large dysplastic cells indicated by arrows, containing nuclear inclusions (×100).* **Right:** *Unlabeled large dysplastic cells* (**short arrows**) *and labeled smaller hepatocytes* (**long arrows**) *(×200).*

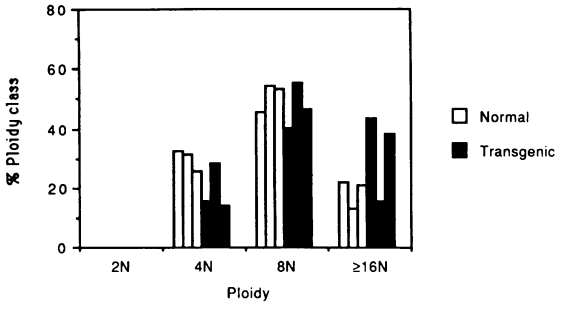

Figure 5. *Ploidy distribution of hepatocytes in older (6- to 8-month-old) normal and TGF-α transgenic mice.* **A:** *Hepatocytes were isolated from the livers of normal* (**open bars**) *and transgenic mice* (**solid bars**)*, and DNA content analyzed by flow cytometry. All livers were grossly normal without evidence of tumors. For each set of three animals, the percentage of the cell population comprising diploid (2N), tetraploid (4N), octaploid (8N), and higher ploidy (≥16N) classes is shown.*

raploid. In contrast, adjacent tissue surrounding the tumors contained only a small proportion (~4%) of diploid cells (Figure 6B). Oval-like cells, if present in the liver, were not included in the ploidy analyses and were removed in the Percoll centrifugation step used for hepatocyte isolation. Although these studies did not directly examine tumor cell lineages, the results are consistent with the hypothesis that the tumors may have developed from cells with low orders of ploidy and not from the large dysplastic hepatocytes present in livers of older mice.

Discussion

During liver development and regeneration as well as in cultured hepatocytes, transient expression of TGF-α is associated with DNA replication.[5,9–13] We show in this work that elevated, persistent TGF-α expression in the liver causes increased proliferative activity of hepatocytes. The high rate of proliferation of hepatocytes of 4-week-old TGF-α transgenic mice appears to be the main cause of the liver enlargement found in these animals and accounts for the elevation of the amount of DNA per gram of tissue in these livers. Cell hypertrophy, which is apparent by histological examination of livers of transgenic mice,[25] may also contribute to the liver enlargement. If hypertrophy were predominant over hyperplasia, it might be expected that the ploidy levels of hepatocytes of young transgenic mice would be higher than those of the controls. However, the opposite is the case, that is, young transgenic mice had a higher proportion of diploid and mononucleated tetraploid hepatocytes

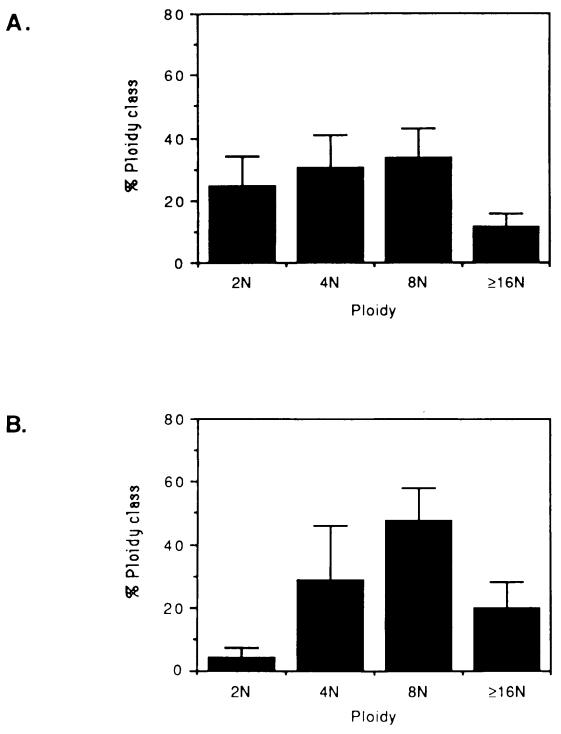

Figure 6. *Ploidy distribution of hepatocytes in tumor-bearing (13-month-old) TGF-α transgenic mice. Flow cytometric analysis of DNA content was performed on hepatocytes from isolated tumors found in three different 13-month-old transgenic mice* (A) *and hepatocytes from the adjacent nontumorous liver parenchyma in the same transgenic mice* (B). *The bars show the average percentage (±SD) of the cell population comprising diploid (2N), tetraploid (4N), octaploid (8N), an higher ploidy (≥16N) classes.*

than CD1 mice of the same age. These findings are consistent with the high replicative activity found in livers of young transgenic mice. All of these data suggest that the persistence of a high level of proliferation in hepatocytes of 4-week-old mice delays the development of polyploidy which normally occurs during the first 2 months of life.[35,36] Similarly, the increase in the proportion of 4N binucleate hepatocytes which takes place during the first month of life in mice[36] appeared to be delayed in transgenic animals.

The constitutive expression of TGF-α altered the liver weight/body ratio in 4-week-old transgenic mice. This observation is of significance because this ratio constitutes an optimal set point for liver growth in normal animals.[1,2] When the set point is disturbed by tissue deficit or excess liver mass, the liver grows or decreases in size, respectively, to restore the normal equilibrium. In young transgenic mice, the liver weight/body weight ratio is set at a higher level than normal, but this abnormally high ratio apparently becomes the optimal value for these animals. This is indicated by the response of the transgenic mice to partial hepatectomy. Although these animals showed a heightened level of DNA synthesis after partial hepatectomy, the relative increase in DNA replication remained proportional to the high basal level of hepatocyte proliferation present before the operation.

The decrease in hepatocyte proliferative activity which normally occurs as the animals age also takes place in transgenic mice. These data as well as the response of the transgenic animals to partial hepatectomy indicate that the constitutive overexpression of TGF-α in the liver initially caused hepatocyte hyperplasia without loss of growth control. Nevertheless, there were major differences between liver growth in transgenic and CD1 mice as they aged. From 1 month to 8 months of age, the hepatocyte proliferative indices in CD1 mice declined by 15 to 20 times, while there was only a four- to sevenfold drop in transgenic mice. Thus, although hepatocyte proliferation declined with age in both control and transgenic mice, the proliferative activity was always higher in transgenic animals relative to CD1 controls. In fact data presented in Table 2 indicate that the relative difference in hepatocyte proliferative activity between CD1 controls and transgenic mice became magnified in 8-month-old animals. It is then surprising that at this age, control and transgenic mice had a similar liver weight/body weight ratio. This finding could be explained by postulating that in transgenic animals which have passed the postnatal liver growth period (approximately 35 days) the higher rate of hepatocyte proliferation was associated with an elevated turnover rate of these cells. The data shown in Figure 1 indicate that this is indeed the case, as shown by the rapid decrease in the number of labeled hepatocytes during the post-label chase period in transgenic but not in control mice. Preliminary evidence also suggest that hepatocyte apoptosis is elevated in the liver of transgenic animals at 3 to 8 months of age. However, we have no clues regarding the mechanisms that may link elevated hepatocyte proliferation caused by TGF-α overexpression and the triggering of apoptosis as a compensatory event which would tend to normalize the liver weight/body weight ratio in transgenic animals.

The events we have described in TGF-α transgenic mice might be explained if we assume that TGF-α acts as a cell cycle progression agent.[1,2] If this is the case, TGF-α overexpression in 1-month-old mice in which hepatocytes normally have the capacity to replicate can maintain a high proportion of cells in the proliferative cycle. As the animals age and hepatocyte proliferative capacity drastically decreases, TGF-α overexpression alone cannot keep the majority of hepatocytes in the cell cycle. Even so, in 8-month-old transgenic animals, a considerable fraction of

hepatocytes continued to exhibit proliferative activity, suggesting that in these cells, replication may have become constitutive. Another possibility is that TGF-α functions as an initiator of hepatocyte proliferation but, in older animals, essential components of the cell cycle machinery become limiting for DNA replication. It is of interest that the expression of TGF-α in the transgenic mice is uneven and that it can be demonstrated by immunohistochemical methods that clusters of hepatocytes have higher expression of the transgene than the surrounding tissue.[25] The level of DNA synthesis in TGF-α positive hepatocytes is three- to fivefold higher than that of hepatocytes that do not show strong TGF-α staining (G.-H. Lee and N. Fausto, unpublished observations). Establishment of constitutive hepatocyte proliferation may be a consequence of anomalies in cell cycle regulation in a subset of TGF-α expressing hepatocytes which resulted from the prolonged cycling of hepatocytes in young TGF-α transgenic mice. Alternatively, TGF-α may have acted preferentially on hepatocytes which were already "spontaneously initiated" and had altered growth properties in very young animals.[25] Our previous work showed that liver tumors that develop in TGF-α transgenic mice frequently overexpress IGF-II and c-*myc* mRNAs in addition to TGF-α.[26] Furthermore, liver tumors appear much more rapidly in TGF-α/c-*myc* double transgenic animals.[27,28] However, we do not know whether overexpression of IGF-II, c-*myc*, or any other gene is preferentially found in replicating hepatocytes of older transgenic mice before the emergence of tumors and whether the overexpression of these genes has a causative role in maintaining the replicating activity of these cells.

Autoradiographs of liver sections from 6- to 8-month transgenic mice infused with ^{3}H-thymidine showed that the very large, often dysplastic hepatocytes present throughout the liver showed little proliferative activity. Most of the replicating cells had no obvious dysplastic features and were generally found in clusters. Although we have not traced the cellular origin of hepatocellular tumors in TGF-α transgenic mice, our results suggest that replicating diploid and tetraploid hepatocytes present in older animals, rather than the large dysplastic cells (which presumably die), are initiated cells from which tumors may originate. Several studies have shown that the expansion of a population of small hepatocytes may be an important step in some types of chemically induced hepatocarcinogenesis[38] and that polyploid cells have reduced proliferative activity in hepatocellular carcinomas.[39]It is also of interest that the induction of hepatocyte proliferation by tumor promoters decreases ploidy levels and the proportion of binucleated cells in the liver.[40,41] This pattern of hepatocyte replication is similar to the cellular changes that occur in livers of TGF-α transgenic mice before the emergence of tumors.

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