Paradoxical Inhibition of c-myc-induced Carcinogenesis by Bcl-2 in Transgenic Mice

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INTRODUCTION

Oncogenesis is a multistep process requiring several genetic alterations that accumulate gradually during tumor development. This process is well illustrated by targeted oncogenesis experiments in transgenic mice (1, 2). Most of the transgenic mice synthesizing activated oncogenic proteins in defined organs develop solitary tumors in a stochastic fashion, which indicates that additional genetic events are required for irreversible cellular transformation (1–3). Early studies on molecular oncogenesis focused on abnormalities of cell proliferation, but it has been recently recognized that the control of cell death and survival are also of paramount significance (for review see Refs. 4 and 5). The first evidence that the inhibition of apoptosis is central to tumor development was obtained in patients with follicular B-cell lymphomas associated with specific t(14:18) chromosomal translocation. The resulting deregulated expression of the bcl-2 gene did not stimulate cell proliferation but promoted the survival of the affected cells until other genetic lesions (frequently c-myc gene rearrangement) led to the more aggressive phenotype (6–8). Bcl-2 was subsequently described as the first member of a growing family of related protein involved in the control of apoptosis (for review see Refs. 9–11). Further evidence for the interrelationship between cell death and transformation came from both in vitro and in vivo studies. The cooperation between c-myc and bcl-2 oncogenes to immortalize different cell lines is now well documented (7, 12, 13). Several models have been developed to examine the role of apoptosis in tumorigenesis (14–18). However, the question of how apoptosis dysregulation contributes to neoplastic transformation remains unanswered. It is presently unclear whether the inhibition of apoptosis is involved only in the later stages of tumor progression or whether earlier changes in apoptosis are critical for promotion of oncogenesis, such as selection of the initiated cell (17, 19).

We have investigated the role of apoptosis in tumor development by studying the effect of bcl-2 gene expression on c-myc-induced hepatocarcinogenesis in transgenic mice. Expression of the c-myc oncogene was targeted to the liver by the regulatory region of the gene encoding L-PK4 (L-PK-c-myc), resulting in the development of hepatocellular carcinomas (20). These mice were crossed with L-PK-bcl-2 mice (21), which are resistant to Fas/Apo/CD95-mediated apoptosis (21). We analyzed tumor development in the double transgenic mice L-PK-c-myc/L-PK-bcl-2 mice. Surprisingly, we observed that not only did coexpression of the bcl-2 and c-myc transgenes not accelerate oncogenesis, but it also inhibited the development of c-myc-induced hepatocarcinogenesis. These results document, for the first time, an in vivo tumor suppressor effect of Bcl-2 during the early stages of hepatic carcinogenesis.

MATERIALS AND METHODS

Transgenic Mice. Transgenic mice were maintained in accordance with the guidelines of the Ministèr e de l’Agriculture et de la Forêt (France) for the care and use of laboratory animals. L-PK-c-myc and L-PK-bcl-2 transgenic mice have been described previously (20, 21). They were obtained by microinjection of either the L-PK-c-myc or L-PK-bcl-2 transgene in fertilized eggs of C57/B6 × DBA hybrids. All transgenic lines were propagated by crossing founders with C57/B6 × CBA hybrid mice. The L-PK-bcl-xL mice were obtained by microinjection of the L-PK-bcl-xL transgene in the same strains of fertilized mouse eggs. The expression of human bcl-xL cDNA (kindly provided by C. B. Thompson, University of Chicago, Chicago, IL) was under the control of the regulatory sequences of the L-PK gene (~5700 to +580 bp; Ref. 21). The TTRp53/DD transgenic mice expressed a truncated form of p53 corresponding to the COOH-terminal domain of the protein (codons 1–13 and 302–390) under the control of the TTR gene transcriptional control region. All mice were continuously fed a carbohydrate-rich diet containing 75% carbohydrates, 15% proteins, and 3% lipids. Tumor incidence analyses were performed by comparison of single and double transgenic littermates.

Northern Blots. Total RNA were extracted from homogenized tissues using the guanidinium thiocyanate procedure (22). Total RNA (15 μg) was separated on 2% (w/v) agarose-formaldehyde gels, transferred to Hybond-N+ nylon membranes (Amersham, Buckinghamshire, United Kingdom), and hybridized with the corresponding probes. Blots were standardized by hybridization with a ribosomal probe (R45).

Western Blots. Liver samples were homogenized in Laemmli sample buffer, boiled for 3 min, and centrifuged for 10 min at 10,000 × g. Samples of extract containing 50 μg of total protein were resolved by 12% (w/v) SDS-PAGE and transferred to a nitrocellulose filter. Membranes were blocked with 5% (w/v) skim milk powder in Tris-buffered saline with 0.05% (v/v) Tween 20. Murine Bcl-xL protein was detected using a rabbit polyclonal anti-Bcl-xL antibody (SC634, 1:1000; Santa Cruz Biotechnology, Santa Cruz, CA). Immunostaining was performed with peroxidase-coupled antirabbit IgG (NA9340, 1:2000; Amersham) and enhanced chemiluminescence (ECL; Am-
Ersham). Blots were standardized by staining with Poncet red and with a rabbit polyclonal anti-annexin V antibody after dehybridization.

**Immunohistochemistry and Liver Histology.** Livers were removed; samples were immediately placed in formalin:acetic acid:alcohol fixative, embedded in paraffin, and sectioned; and the sections were then stained with H&E. The stained sections were examined under a Zeiss microscope and photographed.

**In Vivo BrdUrd Incorporations.** Mice were injected i.p. with BrdUrd (Sigma Chemical Co., St. Louis, MO) dissolved in 0.9% NaCl (100 mg/kg body weight) and sacrificed 2 h later, and their livers were perfused and fixed with 4% formaldehyde. BrdUrd incorporation was then detected with a cell proliferation kit (RP20; Amersham). A total of 1000–3000 hepatocytes were counted for each liver section. The labeling index was defined by the number of BrdUrd-positive cells divided by the total number of cells counted, reported as a percentage.

**Fas Injection and In Situ Detection of Apoptotic Cells: TUNEL Assay.** Mice were injected i.v. with Jo2 anti-Fas antibody (0.5 mg/kg body weight; PharMingen) and sacrificed 3 h later. Liver samples were fixed in 4% paraformaldehyde. The TUNEL assay (23) was performed using the ApopPharMingen) and sacrificed 3 h later. Liver samples were fixed in 4%

| Table 1: Incidence of tumor development in single L-PK-c-myc and double L-PK-c-myc/L-PK-bcl-2, L-PK-c-myc/L-PK-bcl-xL, and L-PK-c-myc/TTRp53DD transgenic mice |
|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|
| Age (months) | Type of lesion | No. of mice | Type of lesion | No. of mice | Type of lesion | No. of mice | Type of lesion | No. of mice |
| 0–0.75 | D | 0/5 | D | 0/4 | ND | ND |
| 0.75–2 | T | 0/10 | T | 0/4 | D | 5/7 | D | 2/3 |
| 2–4 | T | 0/10 | T | 0/7 | T | 0/3 | ND |
| ≥6 | T | 0/31 (64%) | T | 0/82 (36%) | T | 2/2 |
| a | Values represent no. of mice that developed liver lesions (dysplasia or tumor) between birth and 12 months. Livers displaying one or more tumors were scored positive. D, dysplasia; T, tumor; ND, not determined. |
| b | Dysplasia observed during the proliferative stage. |
| c | Dysplasia observed during the quiescent stage. |
| d | The dysplasia observed was more severe than that in the L-PK-c-myc mice. |
| e | P < 0.001. |
| f | Hepatocellular carcinomas occurred earlier, at 7 months, in L-PK-c-myc/L-PK-bcl-xL mice. |

RESULTS

Hepatocellular Carcinoma Progression in L-PK-c-myc Transgenic Mice. L-PK-c-myc transgenic mice developed hepatocellular tumors that resulted from the targeted expression of the c-myc oncogene in the liver (20). The regulatory region of the L-PK gene used in the L-PK-c-myc construct drives expression of the transgene in all hepatocytes and is under the positive control of carbohydrates (20, 24). Sustained expression of the c-myc transgene first led to a high proliferative phase that peaked at 1 month of age, as shown by the presence of numerous mitoses and strong BrdUrd staining (Fig. 1, d and e). Although this period coincided with the mitotically active period of juvenile liver growth, the mitotic activity in L-PK-c-myc mice was 100 times greater (22%) than that of wild-type animal (0.2%; Fig. 1, a and b; see Fig. 4B). This early proliferative stage was associated with the appearance of large dysplastic cells (Fig. 1d; Table 1) and a high apoptotic index (7%), as judged from in situ labeling by the TUNEL technique (Fig. 1f). The dysplastic cells disappeared by the time the mice were 2 months old, probably cleared by apoptosis. Subsequently, from month 2 to month 5, a quiescent phase with less mitotic activity appeared. The mitotic index was only 1/30 of that recorded at 1 month of age (Fig. 1, g and h; see Fig. 4B). Heterogeneous dysplastic changes occurred in hepatocytes during this period, and a progression from slight to severe diffuse dysplasia was observed (Fig. 1j). Dysplasia was intense and associated with a second increase of cell proliferation during month 5 (see Fig. 4B), followed by the emergence of neoplastic foci (Fig. 1j). Liver tumors developed in >80% of L-PK-c-myc mice between 6 and 8 months (Table 1). The tumor incidence in L-PK-c-myc mice was higher in this study than reported previously by Cartier et al. (20) because, in contrast to their study, here, animals were fed a carbohydrate-rich diet continuously, from birth to death. Accordingly, we have observed that the c-myc transgene was highly expressed throughout tumor progression, from month 1 to 11 (see Fig. 4A).

We analyzed the response of L-PK-c-myc-transformed hepatocytes to Fas-mediated apoptosis after i.v. injection of the Jo2 anti-Fas antibody (25), to determine whether transformed hepatocytes had acquired protective properties against apoptosis. Older L-PK-c-myc mice (8 months) were injected with a lethal dose of Jo2, and the percentage survival was determined. Half of the L-PK-c-myc mice (three of six) survived, whereas all their nontransgenic littermates died (25), suggesting that transformed hepatocytes were protected against Fas-mediated apoptosis. The type of hepatocytes protected were identified by killing the mice 3 h after Jo2 injection and analyzing the livers by TUNEL assay. Transformed hepatocytes in neoplastic foci were strongly protected against Fas-mediated apoptosis, whereas non-tumoral surrounding hepatocytes were massively apoptotic (Fig. 2A).

To gain insight into the molecular mechanism involved in this resistance to Fas-mediated apoptosis, we first analyzed Fas expression in the liver of L-PK-c-myc mice. The Fas mRNA level was not altered in the tumor as compared to the nontumoral tissue, whereas a strong overexpression of Bcl-xL, both at the mRNA and at the protein level, was revealed in most of the tumors analyzed (13 of 15; Fig. 2B). The endogenous bcl-2 gene, which is not expressed in the nontumoral liver, was not induced in tumoral sample (data not shown).

We, therefore, infer that tumor progression in L-PK-c-myc transgenic mice was associated with a decreased sensitivity to apoptosis, which could be, at least in part, due to overexpression of the bcl-xL gene.

**Inhibition of c-myc-induced Hepatocarcinogenesis by bcl-2 Transgene Expression.** We then investigated the role of apoptosis in c-myc-dependent hepatocarcinogenesis in more detail. We analyzed the effect of bcl-2 transgene expression on c-myc-induced carcinogenesis. The livers of L-PK-bcl-2 mice express Bcl-2 protein at high level, but with no associated phenotypic changes (21). Single L-PK-c-myc and double L-PK-c-myc/L-PK-bcl-2 littermates were analyzed at different stages of liver carcinogenesis. Surprisingly, we observed a strong inhibition of tumor development in double transgenic mice.
**INHIBITION OF LIVER CARCINOGENESIS BY bcl-2**

Fig. 1. Multistage hepatocarcinogenesis in L-PK-c-myc mice. Liver sections from L-PK-c-myc transgenic mice and nontransgenic mice were analyzed histologically by H&E staining (a, d, g, and j), for cell proliferation by BrdUrd incorporation (b, e, h, and k), and for apoptosis by TUNEL staining (c, f, i, and l). The wild-type mice (a–c) were 1 month old, the time of juvenile liver growth. H&E staining of wild-type hepatocytes (a) revealed BrdUrd-positive cells (b) and TUNEL-positive cells (c). The L-PK-c-myc mice were analyzed at several stages of liver carcinogenesis. At 1 month of age, during the proliferative stage, note the presence of large dysplastic cells (d) associated with a very high proliferative activity (e) and apoptosis (f). At 4 months of age, during the quiescent stage, note the presence of large dysplastic cells (g) but the absence of proliferative (h) or apoptotic (i) cells. The month 8 livers, after the period of neoplastic formation, contained neoplastic foci (j), where there was proliferation (k) and few apoptotic cells (l).

Compared with their single L-PK-c-myc littermates (Fig. 3A). Between 6 and 12 months, 36% (8 of 22) of double transgenic mice developed tumors, compared to 84% (31 of 37) of single L-PK-c-myc mice (Table 1). This difference is highly significant (P < 0.001). The first peak of cell proliferation and apoptosis at 1 month of age and the appearance of diffuse dysplasia during the quiescent stage were similar in both transgenic lines, indicating that Bcl-2 did not inhibit the first histological changes associated with c-myc expression in the liver (Fig. 3B, Table 1). In contrast, expression of bcl-2 appears to strongly inhibit the appearance of liver tumors (Table 1).

**Cell Proliferation and Apoptosis in Single and Double Transgenic Mice.** Because bcl-2 gene overexpression can suppress not only apoptosis (10) but also cell proliferation by preventing quiescent cells from reentering the cell cycle (26, 27), we analyzed the liver proliferative and apoptotic indexes during tumor development in double L-PK-c-myc/L-PK-bcl-2 transgenic mice compared to L-PK-c-myc littermates.

We first established that the two transgenes, c-myc and bcl-2, were expressed independently of each other. Their expressions in double and single transgenic mice were not significantly different and were constant throughout the life of the mouse and during tumor development (Fig. 4A). The bcl-2 transgene expression level was similar in single and double transgenic lines (data not shown). This experiment shows that inhibition of tumorigenesis did not occur by inhibition of c-myc expression as a result of promoter competition.

The first early proliferative peak at 1 month was not altered by bcl-2 gene expression (Fig. 4B), whereas apoptosis seemed to be slightly but not significantly decreased at this time (Fig. 4C). In contrast, the increase in the proliferative and apoptotic activity at the end of the quiescent phase (month 5) was clearly inhibited in the double L-PK-c-myc/L-PK-bcl-2 than in the single L-PK-c-myc mice (Fig. 4, B and C). The difference in proliferation values at month 4 between single and double transgenic mice were not significant (Fig. 4B). These results indicate that bcl-2 gene expression inhibited the second proliferative stage and the associated apoptosis preceding the appearance of neoplastic foci.

**Effect of Other Antiapoptotic Proteins on c-myc-induced Hepatocarcinogenesis.** This unexpected effect of bcl-2 expression on c-myc-induced hepatocarcinogenesis led us to analyze the effect of overexpression of other antiapoptotic genes in these mice. We analyzed bcl-xL, which belongs to the same family as bcl-2 (28), and a dominant-negative form of p53. The L-PK-c-myc mice were crossed with L-PK-bcl-xL transgenic mice overexpressing the bcl-xL gene in the liver, under the control of the rat L-PK regulatory region (29) or with TTRp53DD transgenic mice expressing a dominant-negative p53 in the liver under the control of the TTR gene promoter (30). Neither L-PK-bcl-xL nor TTRp53DD transgenic mice developed any particular phenotype (29, 30).

Table 1 shows that, in contrast to bcl-2, expression of the other antiapoptotic transgenes, truncated p53 and bcl-xL, in L-PK-c-myc transgenic mice, did not counteract tumor development. Instead, hepatocellular carcinomas occurred earlier (7 months) in double L-PK-c-myc/L-PK-bcl-xL mice than in single L-PK-c-myc transgenic littermates (> 8 months; Fig. 5a and data not shown). Dysplasia in month 3 was especially intense in L-PK-c-myc/TTRp53DD mice (Fig. 5b).

Therefore, the antitumorigenic properties of Bcl-2 in L-PK-c-myc mice are not shared by other antiapoptotic molecules, either of the same (Bcl-xL) or of different (truncated p53) families.

**DISCUSSION**

We have examined the role of apoptosis during the multistage process of liver carcinogenesis and found that tumor progression in
L-PK-c-myc transgenic mice is associated with increased protection against apoptosis, in particular, Fas-mediated apoptosis. However, unexpectedly, coexpression of the bcl-2 transgene in the liver counteracted c-myc-mediated hepatocarcinogenesis. Thus, our results indicated for the first time that bcl-2, which was previously demonstrated to cooperate with c-myc in the development of lymphoma (14) and breast cancer (18), can also function as a tumor suppressor gene in the liver.

The transgenic model of liver carcinogenesis induced by c-myc obeys the general rules for targeted hepatocarcinogenesis (31, 32). The requirement for other genetic events in addition to that of the c-myc oncogene is highlighted by the multistage process of liver carcinogenesis and is in agreement with previous work (33). As in other rodent models of hepatocarcinogenesis, the distinct steps of initiation, promotion, and progression must occur (34).

We have shown that the neoplastic foci are resistant to Fas-mediated apoptosis. This is especially noteworthy because c-myc induces apoptosis via the Fas pathway (35). The molecular mechanisms of this resistance are not yet known. Fas gene expression was not downregulated in the tumors of L-PK-c-myc mice, but it is always possible that the Fas signaling pathway was deregulated, as has been proposed in human hepatocellular carcinoma (36, 37). However, the overexpression of the bcl-xL gene is likely to contribute to the resistance of neoplastic hepatocytes to Fas-mediated apoptosis, as recently described in Jurkat cells (38).

In striking contrast to the expected role of Bcl-2 in apoptosis suppression during tumor development, coexpression of the antiapoptotic bcl-2 transgene in the liver of L-PK-c-myc transgenic mice not only does not result in acceleration of the oncogenic process but also strongly inhibits the appearance of liver tumors.

Because overexpression of the bcl-2 gene can suppress both apoptosis (for review see Refs. 9, 11, 39, and 40) and cell proliferation by preventing quiescent cells from reentering the cell cycle (7, 26, 27, 41), there are two possible explanations for the bcl-2-dependent inhibition of c-myc-induced hepatocarcinogenesis. (a) The inhibition of growth by bcl-2 may inhibit proliferation of the initiated cells and, thus, prevent the progression towards malignant phenotypes. This is supported by the observation that overexpression of the bcl-2 gene in quiescent cells retards entry into the cell cycle but does not affect growth of cells that are continuously cycling (27, 41). Initiated cells are believed to appear during the quiescent dysplastic stage, after the initial proliferation peak (33). (b) Apoptosis in the earlier stage of

![Fig. 2. Resistance of L-PK-c-myc-transformed hepatocytes to Fas-mediated apoptosis.](image)

A. TUNEL analysis of a liver section from an 8-month-old L-PK-c-myc mouse, 3 h after the i.v. injection of Jo2 anti-Fas antibody. There was no staining of transformed hepatocytes in the neoplastic foci, whereas the surrounding normal hepatocytes were heavily stained, indicating massive apoptosis. B. Bcl-xL, bcl-2, overexpression analysis by Western blotting. Each tumor sample (Lanes T) analyzed showed strong overproduction of Bcl-xL compared for each mouse to the nontumoral surrounding liver (Lanes N). Blots were standardized with anti-annexin V antibody. C. m Fas expression was analyzed by Northern blotting. The amount of Fas in nontumoral (Lanes N) and tumoral (Lanes T) livers was similar for each mouse.

![Fig. 3. Inhibition of tumor development in L-PK-c-myc/L-PK-bcl-2 double transgenic mice.](image)

A. livers isolated from 8-month-old L-PK-c-myc, L-PK-c-myc/L-PK-bcl-2, and nontransgenic mice. Tumors and nodules were found only in the liver from the single L-PK-c-myc transgenic mouse. B. liver sections from double transgenic mice aged 1 and 4 months. The high proliferative and apoptotic activities in the L-PK-c-myc mice were also found in the double transgenic mice (a and b). The liver of a 4-month-old double transgenic mouse was dysplastic (c). HES, H&E stain.
Apoptosis persisted in tumoral (T) only in apoptosis decreased in the quiescent stage. At 5 months of age, apoptosis was increased (5%) in mice at 1 month of age, than in their wild-type littermates (0.1%). Then bcl-2 counted. Apoptotic activity was higher in both expressed as the number of TUNEL-positive cells divided by the total number of cells TUNEL staining (three animals by transgenic line and by age). The apoptotic index is, apoptosis in hepatocytes of transgenic mice. The apoptotic activity was determined by transgenic mice (16%) and less in the nontumoral surrounding areas (3.9%).

Expression of c-myc/L-PK was scored on stained sections and is expressed as a percentage (number of BrdUrd-negative cells divided by the total number of cells from the same mouse (Lanes 10 and 11 and age (months) and 12 and 13). There were two sizes of c-myc and bcl-2 mRNA: the smaller one is due to the correctly spliced transgene, and the larger one to an unspliced form containing the first intron (500 bp) of the rat L-PK gene. Northern blots were standardized with a ribosomal probe (R45). B, mitotic activity in the hepatocytes of transgenic mice (three animals by transgenic line and by age). The mitotic activity was scored on stained sections and is expressed as a percentage (number of BrdUrd-positive cell divided by the total number of cells counted). The mitotic indexes for the L-PK-c-myc and L-PK-c-myc/L-PK-bcl-2 transgenic mice were similar from 0.5 to 4 months, with a high peak of proliferation (22 and 28%) at 1 month, followed by a quiescent stage with low mitotic activity. Proliferation increased again at 5 months (7.8%) only in L-PK-c-myc hepatocytes and not in the double L-PK-c-myc/L-PK-bcl-2 mice (0.55%; *, p < 0.05). Hepatocytes proliferated strongly in the tumoral regions (T) of L-PK-c-myc transgenic mice (16%) and less in the nontumoral surrounding areas (3.9%). C, apoptosis in hepatocytes of transgenic mice. The apoptotic activity was determined by staining (three animals by transgenic line and by age). The apoptotic index is expressed as the number of TUNEL-positive cells divided by the total number of cells counted. Apoptotic activity was higher in both L-PK-c-myc (7%) and L-PK-c-myc/L-PK-bcl-2 (5%) mice at 1 month of age, than in their wild-type littermates (0.1%). Then apoptosis decreased in the quiescent stage. At 5 months of age, apoptosis was increased only in L-PK-c-myc hepatocytes (2.5%), in parallel with the resumption of proliferation. Apoptosis persisted in tumoral (T) tissue (2.55%).

Fig. 4. Apoptosis, proliferation, and transgene expression in L-PK-c-myc and L-PK-c-myc/L-PK-bcl-2 transgenic mice. A, c-myc and bcl-2 transgene expression in transgenic mice. A representative Northern blot illustrates c-myc and bcl-2 transgene expression in various sample (Lanes N, nontumoral liver; Lanes T, tumoral liver) from single L-PK-c-myc (myc) and double L-PK-c-myc/L-PK-bcl-2 (myc/bcl2) transgenic mice. Expressions of c-myc mRNA in the single L-PK-c-myc (Lanes 2, 4, 6, 8, 9, 12, and 13, left to right) and double L-PK-c-myc/L-PK-bcl-2 (Lanes 1, 3, 5, 7, 10 and 11) mice were not significantly different (15 samples of single and double transgenic mice were analyzed). Bcl-2 expression was not down-regulated in the double transgenic mice (Lanes 1, 3, 5, 7, 10, and 11). Expression of c-myc and Bcl-2 was also similar in nontumoral and tumoral livers samples from the same mouse (Lanes 10 and 11 and 12 and 13). There were two sizes of c-myc and bcl-2 mRNA: the smaller one is due to the correctly spliced transgene, and the larger one to an unspliced form containing the first intron (500 bp) of the rat L-PK gene. Northern blots were standardized with a ribosomal probe (R45). B, mitotic activity in the hepatocytes of transgenic mice (three animals by transgenic line and by age). The mitotic activity was scored on stained sections and is expressed as a percentage (number of BrdUrd-positive cell divided by the total number of cells counted). The mitotic indexes for the L-PK-c-myc and L-PK-c-myc/L-PK-bcl-2 transgenic mice were similar from 0.5 to 4 months, with a high peak of proliferation (22 and 28%) at 1 month, followed by a quiescent stage with low mitotic activity. Proliferation increased again at 5 months (7.8%) only in L-PK-c-myc hepatocytes and not in the double L-PK-c-myc/L-PK-bcl-2 mice (0.55%; *, p < 0.05). Hepatocytes proliferated strongly in the tumoral regions (T) of L-PK-c-myc transgenic mice (16%) and less in the nontumoral surrounding areas (3.9%).

C, apoptosis in hepatocytes of transgenic mice. The apoptotic activity was determined by TUNEL staining (three animals by transgenic line and by age). The apoptotic index is expressed as the number of TUNEL-positive cells divided by the total number of cells counted. Apoptotic activity was higher in both L-PK-c-myc (7%) and L-PK-c-myc/L-PK-bcl-2 (5%) mice at 1 month of age, than in their wild-type littermates (0.1%). Then apoptosis decreased in the quiescent stage. At 5 months of age, apoptosis was increased only in L-PK-c-myc hepatocytes (2.5%), in parallel with the resumption of proliferation. Apoptosis persisted in tumoral (T) tissue (2.55%).

Fig. 5. Tumor development in L-PK-c-myc/L-PK-bcl-x L or L-PK-c-myc/TTRp53DD double transgenic mice. a, H&E staining of a liver section from a 7-month-old L-PK-c-myc/L-PK-bcl-x L transgenic mouse, showing a well-differentiated hepatocellular carcinoma. b, H&E staining of a liver section from a 3-month-old L-PK-c-myc/TTRp53DD transgenic mouse, revealing severe dysplasia.

carcinogenesis may be necessary to increase the selective pressure on the initiated cells to favor the emergence of proliferative, apoptosis-resistant cells. This is supported by the finding that coexpression of two proapoptotic genes, transforming growth factor β1 and the hepatitis B virus X, potentiates c-myc-induced hepatocarcinogenesis (42–44). Thus, the overexpression of the bcl-2 gene may either directly inhibit the growth of the initiated cells and/or indirectly impede their selection.

We have recently shown that the expression of the bcl-2 gene does not inhibit hepatocarcinogenesis induced by the SV40 large T antigen; rather, it accelerates hepatocarcinogenesis. Accordingly, hepatocarcinogenesis induced by the SV40 large T antigen is dramatically different from the hepatocarcinogenesis induced by c-myc. The hepatocytes were never quiescent in these mice and had high mitotic activities throughout the lives of the mice as has been described previously in similar model (45). Consequently, because bcl-2 only inhibits the proliferation of quiescent cells but not that of continuously cycling cells (27, 41), Bcl-2 cannot be antiproliferative in SV40-T antigen transgenic mice. This also explains why Bcl-2 would not block the first peak of proliferation, at 1 month of age, which coincides with juvenile growth when hepatocytes are continuously cycling. Thus, our results, in agreement with previous data (7, 26, 27, 41, 46, 47), suggest that bcl-2 can function as a tumor suppressor gene by inhibiting the cell cycle. This effect appears to be specific to bcl-2 and to depend on the cellular context (our results and Ref. 46).

The notion that Bcl-2 could function as a tumor suppressor is important because overexpression of bcl-2 may explain why some lymphomas are not very malignant and why the prognosis for some breast and colorectal tumors is better (48–52). A shift from synthesizing Bcl-2 to the Bcl-xL protein occurs during the progression of colorectal tumors (51), and the loss of bcl-2 expression is correlated with high tumor grade in breast cancer (50). Finally, elevated bcl-xL expression is now recognized to be a frequent acquired antiapoptotic mechanism in cancer cells (51, 53). If our assumption is correct, then
it can be hypothesized that mutations of the bcl-2 gene affecting its capacity to inhibit the cell cycle should be selected for during tumor progression.

ACKNOWLEDGMENTS

We thank Patrick Mayeux and Chantal Cremesi for helpful discussions and Arlette Dell’Amico, Isabelle Loutouge, Hervé Gendrot, and Nathalie Bâ for technical assistance.

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