The rise and fall of apoptosis during multistage tumorigenesis: down-modulation contributes to tumor progression from angiogenic progenitors

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In a mouse model of multistage tumorigenesis of islet β-cells, apoptosis was activated concomitant with T-antigen oncogene-induced cell proliferation, further increased in the angiogenic stage, and markedly reduced in solid tumors. Crosses to p53-null mice confirmed this stage-specific variation as a p53-independent apoptotic process. Several apoptosis regulators were expressed, of which bcl-x_L was up-regulated in tumors. When overexpressed throughout the pathway, bcl-x_L protected most oncogene-expressing cells from apoptosis, enhancing progression from angiogenic progenitor to tumor without affecting earlier transitions. Further, two classes of solid tumor are described, distinguished by size and apoptotic incidence, implicating apoptosis regulation in expansive tumor growth. Thus, down-modulation of apoptosis selectively contributes to late steps in a tumorigenesis pathway.

Apoptotic cell death is characterized by distinctive morphological changes in the dying cell, including membrane blebbing, cytoplasmic and nuclear condensation, chromatin fragmentation, and formation of apoptotic bodies (Wyllie 1980). Apoptosis plays important roles in a variety of developmental events and in the control of tissue homeostasis (Kerr et al. 1987; Oppenheim 1991; Raff 1992). It is an active cellular process that is genetically controlled, thus making it amenable to genetic manipulation. In recent years, there has been an avalanche of information on the genes involved in the regulation of apoptosis (White 1996). It is increasingly apparent that regulation of apoptosis may play significant roles in disease processes, including cancer (Thompson 1995).

The importance of apoptosis in neoplastic transformation was realized in retrospect on the basis of the observation that the most common genetic change in human B-cell follicular lymphoma was a translocation that juxtaposed the bcl-2 gene with the immunoglobulin heavy chain gene (Eμ-bcl-2) (Bakhshi et al. 1985; Cleary and Sklar 1985; Tsujimoto et al. 1985). Subsequently, it was found that bcl-2 does not stimulate proliferation, but rather confers survival upon cells (Vaux et al. 1988; Hockenberry et al. 1990). Thus, this novel class of oncogene regulates cell death (Korsmeyer 1992). Further evidence for an interrelationship between cell death and cell transformation came from cell-culture studies indicating that expression of certain growth-stimulatory oncogenes leads to a paradoxical consequence of cell proliferation and cell death under conditions that restrict growth (Evan et al. 1992; White et al. 1994; White 1996). In one example, transformed epithelial cells expressing adenovirus E1A accumulate slowly as a result of concordant cell death, which can be ameliorated by the presence of the death-protecting E1B 19K protein or bcl-2 (White et al. 1994; White 1996). Thus, complete transformation of a cell may require both increased proliferative capacity and protection from apoptosis.

Studies of animal models have also implicated apoptosis as a component of cancer development. Transgenic mice expressing both c-myc and bcl-2 develop tumors with a significantly reduced latency period (Strasser et al. 1990) compared with transgenic animals harboring just the c-myc transgene (Adams et al. 1985) or an Eμ-bcl-2 hybrid minigene (McDonnell and Korsmeyer 1991). It is noteworthy that tumors in the double transgenic animals were of a different cell type from those in single transgenic mice. Development of these tumors presumably results from synergy between protection from cell death conferred by bcl-2 and the growth stimulatory function of c-myc. This and other studies (Howes et al. 1994; Pan and Griep 1994; Symonds et al. 1994; Ziegler et al. 1994) suggest that modulation of apoptosis is im-

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portant for the growth of tumors. It is presently unclear, however, whether apoptosis is differentially regulated in the stages of the multistep tumorigenesis pathway that characterize most cancers: Does a critical change(s) in apoptosis occur in the preneoplastic stages, or is it important only in the later stages of tumor progression?

Induction of apoptosis can be dependent on or independent of p53 tumor suppressor function, according to cell type and nature of stimuli. Although most studies have focused on the involvement of p53 in regulating apoptosis, it is clear from several reports that a p53-independent pathway of apoptosis exists (Berges et al. 1993; Clarke et al. 1993; Lowe et al. 1993b; Strasser et al. 1994). In fact, many cell types are capable of executing apoptosis during embryogenesis through a route independent of p53, because p53-null mice develop normally and are generally able to maintain normal tissue homeostasis (Donehower et al. 1992). Thus, p53-independent apoptosis is likely to be a major component of most cells' repertoire for inducing cell suicide, the dysregulation of which could be important for diseases such as cancer.

To dissect the contribution of different events to multistage carcinogenesis, we studied a transgenic mouse model in which the regulatory region of the rat insulin-II gene targets expression of the SV40 large T-antigen (Tag) oncogene to the β cells of the pancreatic islets (the RIP-Tag lines) (Hanahan 1985). The Tag oncoprotein exerts its oncogenic effect in part through binding to, and inactivation of, two tumor suppressor proteins, pRb and p53 (Ludlow 1993). The expression of Tag in RIP-Tag mice commences at embryonic day 9 at the onset of insulin expression in the pancreatic diverticulum and persists in the β cells of all islets throughout adulthood (Alpert et al. 1988). Demonstrating a remarkable penetrance of the neoplastic phenotype, 100% of the mice in two transgenic families, RIP1-Tag2 (R1T2) and RIP3-Tag2 (R3T2), develop a few islet cell carcinomas by 14 weeks of age (Hanahan 1985). Tumor progression has been shown to be multistep, with several preneoplastic stages identified by temporal, histological, and statistical criteria. Despite the embryonic onset of Tag expression, the first change is not observed until the mice are 4–6 weeks of age, when individual islets switch on aberrant proliferation, characterized by the appearance of mitotically active cells scattered uniformly throughout the islet (Teitelman et al. 1988). Although historically referred to as hyperplastic or hyperproliferative, these islets are populated with cells showing the hallmarks of cellular malignancy; as such, these numerous nodules are analogous to the multifocal carcinoma in situ (CIS) lesions seen in many epithelial cancers. By 8–10 weeks of age, some islets acquire an angiogenic phenotype, as determined by their activation of new blood capillary growth and angiogenic activity in vitro (Folkman et al. 1989). Finally, by 11–12 weeks of age, a few solid, encapsulated tumors emerge. Statistical analysis of the islet types during the Rip-Tag tumorigenesis pathway reveals that, of the ~400 islets in the pancreas, eventually 50–75% become hyperplastic, 10% become angiogenic, and 1–2% develop into solid tumors. The multistep nature of the

pathway indicates that, despite its expression in all β cells of all the islets, Tag expression alone is insufficient for tumor development. Thus, further changes need to occur, in conjunction with Tag expression, for the elaboration of tumors. We have found that regulation of apoptotic cell death is one of these critical changes.

The first clue that regulation of apoptosis influenced tumorigenesis came from studies of insulin-like growth factor 2 (Igf-2) in RIP-Tag mice. Igf-2 was found to be expressed concomitant with the switch to hyperplasia and thus was a candidate factor responsible for the hyperplastic switch. When its functional contribution to tumor development was assessed by intercrossing the RIP-Tag mice into an Igf-2-null background, however, the most obvious phenotype was a dramatic reduction in tumor size; Igf-2-null tumors had the same mitotic index as Igf-2 wild-type tumors, but exhibited a fivefold increase in the incidence of apoptosis. These data pointed to a survival, rather than a mitogenic, function for Igf-2. Furthermore, it was clear that although Igf-2-positive tumor cells exhibited a lower propensity to undergo cell suicide than Igf-2 null tumors, apoptosis was nonetheless discernible in the wild-type tumors (Christofori et al. 1994; Naik et al. 1994). Taken together, these observations suggested a potential role for apoptosis regulation in tumorigenesis, motivating the current study.

We have investigated the regulation and role of apoptosis, specifically that which is independent of p53, in multistage carcinogenesis. We report that apoptosis is regulated dynamically in RIP-Tag multistep tumor development, and that its induction is contemporaneous with deregulation of cell proliferation in Tag-expressing cells. Functional tests reveal that down-modulation of apoptosis contributes to the frequency of progression from the angiogenic islet stage to solid tumor lesions.

Results

Apoptosis is dynamically regulated in multistep tumorigenesis

To assess the incidence of apoptosis in the RIP-Tag tumorigenesis pathway, apoptotic cells were visualized by a modified TdT-mediated dUTP–digoxigenin nick-end labeling (TUNEL) method (Surh and Sprent 1994). Tissue sections from 7- to 14-week-old mice were examined. Most analysis was done on 10- to 14-week-old mice, because pancreas sections from these animals had islets of all stages, thus allowing assessment of cell death in all islet types within the same tissue sections. This strategy ensured that the differences in apoptotic incidence were not attributable to differences in sensitivity of the TUNEL assay resulting from variations in tissue fixation between sections.

"Normal" Tag$^+$ islets from RIP1Tag2 (R1T2) mice (Fig. 1Aa,B), as well as nontransgenic islets (data not shown), generally did not exhibit a significant amount of cell death. In contrast, apoptosis was clearly detected in hyperplastic islets (Fig. 1Ac,B), and reached maximal frequency in angiogenic islets (Fig. 1Ae,B). In tumors (Fig.
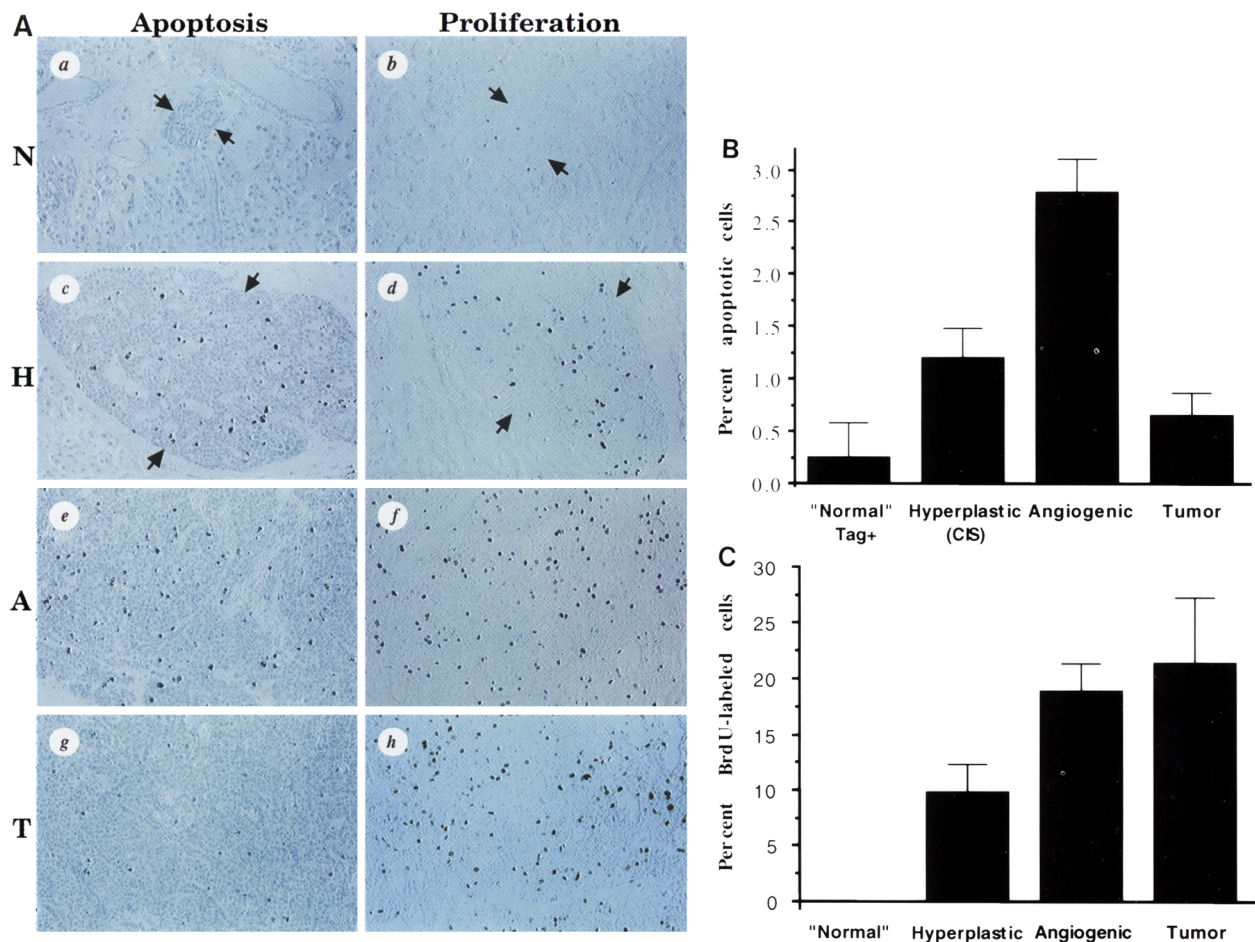
Figure 1. Assessment of apoptotic and proliferation indices in RIP-Tag multistep tumorigenesis pathway. (A) Apoptotic cells were assessed by TUNEL staining (a,c,e,g), and proliferating cells by BrdU staining (b,d,f,h). Islets of all four stages from one 15-week-old animal are shown. (a,b) Normal; (c,d) hyperproliferative; (e,f) angiogenic islets; and (g,h) tumors. The boundaries of normal and hyperproliferative islets are marked by arrows; e,f,g,h show part of a single angiogenic islet or tumor. N, normal Tag+ islets; H, hyperproliferative islets (carcinoma in situ); A, angiogenic islets; and T, large end-stage tumor. Magnification, 80×. The indices of apoptotic (B) and proliferating cells (C) are expressed as percentage of total cells within an islet that are positive by TUNEL or BrdU staining, respectively. For apoptotic index, the average of median values from three independent rounds of experiments involving 98 normal, 44 hyperplastic, 41 angiogenic islets, and 15 large tumors from 15 R1T2 animals are shown. For proliferation index, the average values for 23 "normal," 13 hyperplastic 17 angiogenic islets, and 8 tumors from four R1T2 mice are shown.

1Ag,B), however, apoptotic incidence was markedly reduced, by approximately fourfold compared with that of the angiogenic stage. A second independent RIP-Tag line of transgenic mice, RIP3Tag2 (R3T2), also displayed the same pattern and incidence of apoptosis throughout its multistep tumor development (data not shown).

To test the hypothesis that oncogene-induced deregulation of DNA synthesis (cell proliferation) can lead to apoptosis, we determined the mitotic index, as measured by bromodeoxyuridine (BrdU) incorporation, of the various stages. As reported previously (Teitelman et al. 1988), in the transition from normal to hyperplasia, islets convert from a quiescent state to one that exhibits significant mitotic activity (Fig. 1Ab,d,C). The mitotic index continued to increase and peaked in angiogenic islets, and remained essentially the same in tumors (Fig. 1Af,h,C). It is notable that the proliferating cells were

distributed uniformly, as were apoptotic cells, throughout islets of all stages. The change in mitotic activity of the preneoplastic islets clearly parallels the induction of cell death in these stages. These data are consistent with a scenario whereby deregulation of cell proliferation is accompanied by apoptosis.

Apoptosis in RIP-Tag tumorigenesis is not p53-dependent

A number of studies have demonstrated a critical requirement for p53 function in apoptosis (Clarke et al. 1993; Lowe et al. 1993a,b). In one study involving a mouse model of brain tumorigenesis, apoptosis in tumors was abrogated only when p53 was bound by Tag (Symonds et al. 1994). Thus, our observation of significant levels of apoptosis in the RIP-Tag pathway was

rather unexpected. To assess the p53 requirement of the cell death detected in RIP-Tag islets more definitively, we examined R1T2, p53 homozygous null mice. Two issues motivated this experiment. First, although it is known that Tag binds to p53, we have shown previously that residual free, unbound p53 remained within the Tag-expressing cells (Efrat et al. 1987). It is thus conceivable that this p53 species accounts for the apoptosis observed. Second, it is possible that the high incidence of apoptosis in angiogenic islets resulted from hyperinduction of p53 or reduction in Tag levels in this particular stage of tumorigenesis.

Tissue sections of R1T2/p53$^{+/+}$ and R1T2/p53$^{-/-}$ mice were analyzed by the TUNEL assay. The incidence of apoptosis was determined for both hyperplastic and angiogenic islets. As shown in Figure 2, the apoptotic index in angiogenic islets was unaffected by the p53 genotype; the incidence of apoptosis in hyperplastic islets (not shown) was also similar between the two types of mice. These data demonstrate that the apoptosis observed in islet cells does not require p53 function, and thus is independent of p53.

Apoptosis regulatory genes are expressed in RIP-Tag islets

The products of many genes have been found to have apoptosis regulatory functions; these can be classified as transcellular (Igf-2, Fas ligand, etc.) and intracellular. Among the best-studied of the intracellular regulators is the *bcl-2* family of genes. In preliminary RNA-PCR analyses, we did not detect bcl-2 expression in normal control or RIP-Tag islet cells, whereas two other *bcl-2* family members, *bcl-x$_L$* (Boise et al. 1993) and *bax* (Oltvai et

al. 1993), and a gene encoding a bcl-2-associated protein, *bag-1* (Takayama et al. 1995), were expressed (data not shown). Therefore, we employed RNA in situ hybridization to further assess the expression of these genes during tumorigenesis.

In R1T2 animals, the "death protector" bcl-x$_L$ was found to be expressed at very low levels in normal (Fig. 3a), hyperplastic (Fig. 3d), and angiogenic (Fig. 3g) islets, but was clearly up-regulated in tumors (Fig. 3j). By use of a probe comprising the region that is deleted in the death-inducing bcl-x$_s$ splice variant (Boise et al. 1993), the expression of bcl-x was confirmed to be that of bcl-x$_L$ (data not shown). Moreover, bcl-x$_s$ was not detected by RNA-PCR (data not shown). Expression of the death inducer bax, on the other hand, was high, but relatively constant, throughout the tumorigenesis pathway (Fig. 3b,e,h,k). Similarly, there was a very high and constant level of expression of bag-1 in all stages of tumor development (Fig. 3c,f,i,l).

Modulation of apoptosis by bcl-x$_L$

Our analysis of apoptosis regulatory genes in RIP-Tag islets revealed that both positive (bcl-x$_L$ and bag-1) and negative (bax) intracellular regulators of apoptosis are expressed in the RIP-Tag islet cells. These results suggest that the observed cell death could be attributed to interactions among these and other apoptosis regulatory proteins, with the ratio of these interacting proteins being a determinant of the fate of the cells. Therefore, we sought to determine if overexpression of bcl-x$_L$ could influence the apoptotic phenotype in RIP-Tag islets. The fact that a gene knockout of bcl-x$_L$ results in embryonic lethality (Motoyama et al. 1995) precluded examination of the effects of its loss of function on tumorigenesis and led us to assess its importance by deliberately overexpressing bcl-x$_L$ throughout the pathway.

A transgene was created by placement of the human bcl-x$_L$ cDNA under control of a 9.5-kb rat insulin II 5' regulatory region (Fig. 4A). Two independent transgenic lines were generated, RIP7-bcl-x$_L$1 and RIP7-bcl-x$_L$2. Each was shown to express bcl-x$_L$ at levels greater than 20-fold above endogenous levels, as assessed by immunostaining, in virtually all islets of the mice as early as 5 weeks of age (the youngest age examined) (data not shown). The RIP7-bcl-x$_L$ (R7bx$_L$) mice were healthy and did not exhibit any obvious phenotype through one year of age.

To assess the effect of bcl-x$_L$ overexpression on apoptosis in the RIP-Tag mice, double-transgenic R1T2/R7bx$_L$ mice were generated by use of the RIP7-bcl-x$_L$1 line, and the apoptotic indices compared against R1T2 littermates. In both hyperplastic and angiogenic islets, the two stages exhibiting the most prominent apoptosis in RIP-Tag mice, cell death was greatly reduced in R1T2/R7bx$_L$ mice (Fig. 4B). Most significant, in R1T2/R7bx$_L$ angiogenic islets, there was a fourfold reduction to the level of apoptosis normally seen in R1T2 end-stage tumors. Thus, in R1T2/R7bx$_L$ mice, the incidence of apo-
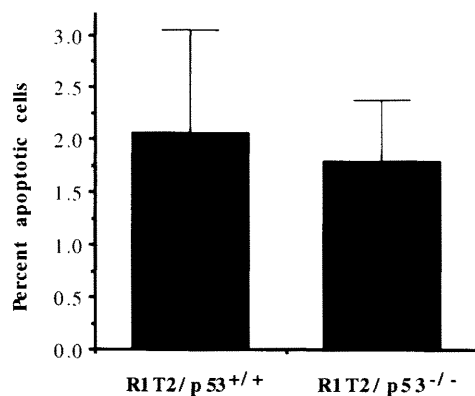

Figure 2. Assessment of p53 requirement for apoptosis in the RIP-Tag pathway. Apoptosis in the angiogenic islets of R1T2/p53$^{+/+}$ and R1T2/p53$^{-/-}$ littermate 10-week-old animals was assessed by TUNEL staining. The index was determined as described in the legend for Fig. 1. The mean values from 19 islets of 3 R1T2/p53$^{+/+}$ and 27 islets of 3 R1T2/p53$^{-/-}$ animals are presented. The variance implied in the error bars reflects the continuum of progression within the broad category of angiogenic lesions, which range from early angiogenic to fully angiogenic, as described previously (Parangi et al. 1995).
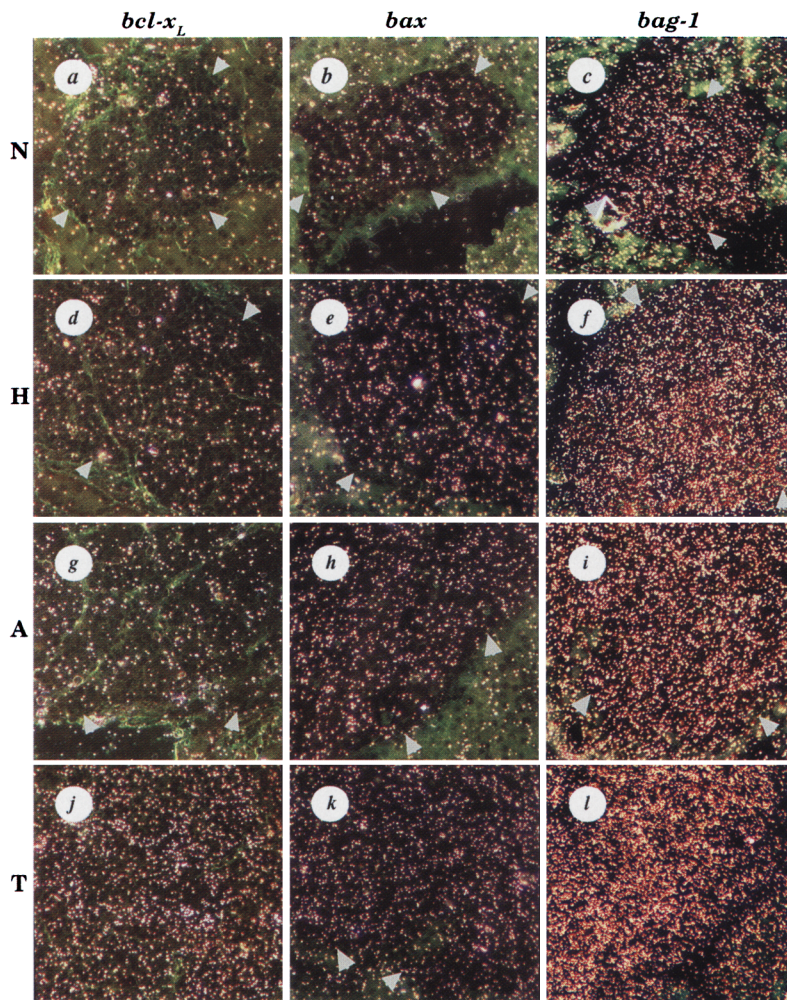
Figure 3. RNA in situ hybridization analysis of bcl-x$_L$, bax, and bag-1 expression in RIP-Tag tumorigenesis. Expression of bcl-x$_L$ (a,d,g,j), bax (b,e,h,k), and bag-1 (c,f,i,l) in R1T2 islets of all four stages was assessed by RNA in situ hybridization. (a–c) Dark-field photomicrographs of normal islets; (d–f) hyperplastic islets; (g–i) angiogenic islets; and (j–l) tumors. For hyperplastic, angiogenic, and tumors, only a portion of the islet is shown; arrowheads demarcate boundaries of islet cell and exocrine tissue. To allow reliable quantitative comparison, expression of each gene is shown for islets of all stages from the same tissue section. (N) normal Tag$^+$ islets; (H) hyperproliferative islets (carcinoma in situ); (A) angiogenic islets; and (T) large end-stage tumor. Magnification 100×.

ptosis is restricted at all stages to the baseline frequency normally observed only in tumors.

Down-regulation of apoptosis acts at a late step in tumor progression

To assess the effect of down-modulation of cell death in multistep carcinogenesis, the incidence of angiogenic islets in 11-week-old R1T2/R7bx$_L$ double-transgenics and R1T2 single-transgenic littermates was compared. Mice from both genotypes exhibited a similar incidence of angiogenic islets (Fig. 5A). Thus, the data demonstrate that down-regulation of apoptosis does not significantly alter the frequency of the angiogenic stage of this multistage pathway, a stage characterized by a high apoptotic index.

In contrast to the lack of effect on the formation of angiogenic islets, the tumor incidence in R1T2/R7bx$_L$ mice was found to be significantly higher than R1T2 single-transgenic littermates. The number of tumors in 11-week-old mice (an age when tumors normally begin to develop) was determined. Tumor number was expressed as a percentage of the number of angiogenic islets in individual mice, to account for the range in number of angiogenic islets that were present in individual mice (Fig. 5B). On average, the incidence of progression was about 2.5-fold that of R1T2 single transgenic animals (Fig. 5B, Table 1). Thus, down-regulation of apoptosis has a critical impact on the conversion from the late preneoplastic state of angiogenic islets to that of islet cell carcinoma.

To determine if the increased incidence of solid tumor formation persists throughout the lives of these mice, end-stage (14- to 15-week-old) mice were examined. Again, the tumor incidence in R1T2/R7bx$_L$ mice was significantly higher than in R1T2 mice, showing a 2.6-fold increase (Fig. 5C,D, Table 1). The double-transgenic mice often had a striking increase in tumor number; in some instances, individual mice developed >45 tumors (these pancreases had a grape-like appearance), whereas the highest number of tumors ever observed in a R1T2 single transgenic animal was 17. The range in tumor numbers observed among animals of each genotype likely reflects the range in number of angiogenic islets that developed in these mice (see Fig. 5A). Nevertheless, the range in tumor number per mouse observed in R1T2/R7bx$_L$ double-transgenic mice was statistically signifi-
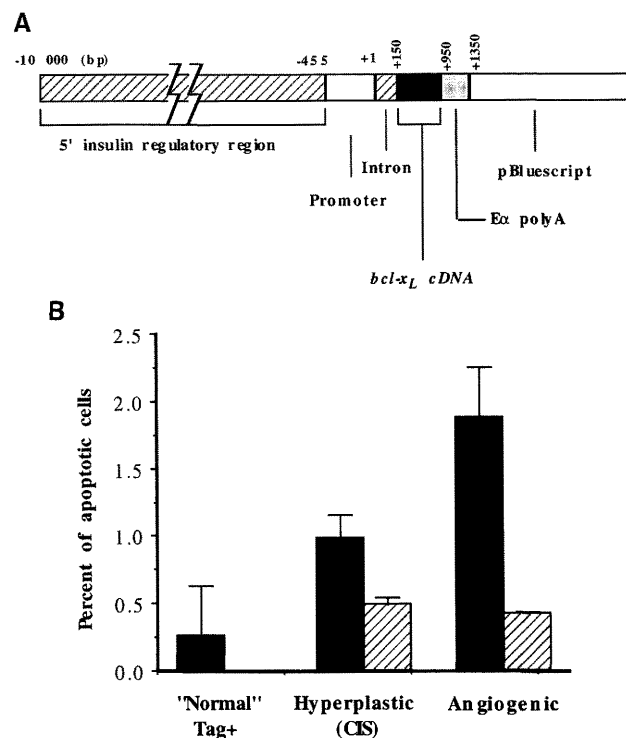
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A

B

Figure 4. Targeted overexpression of bcl-x_L in the pathway. (A) The gene used to generate RIP7-bcl-x_L (R7bx$_L$) transgenic mice consisted of a human bcl-x_L cDNA controlled by 9.5-kb of the 5' rat insulin regulatory region, the first intron of the rat insulin gene and the MHC class II Ea terminator sequence. Total length of transgene was about 14 kb. (B) The effect of bcl-x_L overexpression on islet cell apoptosis was assessed by TUNEL staining of normal, hyperplastic, and angiogenic islets from R1T2 mice (filled bars), and compared with that of R1T2/R7bx$_L$ double-transgenic animals (hatched bars). The average of median apoptotic values from two independent rounds of analysis involving 98 normal, 27 hyperplastic and 40 angiogenic islets from 3 R1T2/R7bx$_L$ mice and 55 normal, 30 hyperplastic, and 33 angiogenic islets from three littermate R1T2 animals are shown.

cantly higher and nonoverlapping with that of the R1T2 animals (Fig. 5D). Preliminary analysis of a second line, R3T2, again revealed a similar increase in tumor incidence of R3T2/R7bx$_L$ mice as compared with R3T2 single transgenics (data not shown). Thus, persistent downregulation of apoptotic cell death at all stages of neoplasia results in increased propensity to progress to solid tumors.

Apoptosis is down-regulated in two discrete steps in solid tumors

In the R1T2/R7bx$_L$ double-transgenic mice, we were unable to rigorously compare cumulative tumor volume because of the increased propensity of these mice to die of insulinoma-induced hypoglycemia. Therefore, we used an alternative method to assess the effect of apoptosis on tumor growth. We reasoned that if down-regulation of apoptosis is important for tumor growth, then

apoptotic incidence would be inversely correlated with tumor size in R1T2 single-transgenic mice.

Pancreas sections from 10- to 15-week-old R1T2 mice were examined for apoptosis with the TUNEL assay. These sections generally presented multiple tumors that could be categorized as nascent small tumors and large mature tumors. The apoptotic incidence of these tumors, as well as angiogenic islets in the same sections, was determined. The data showed a clear inverse correlation between apoptosis and tumor size (Fig. 6). Unexpectedly, the incidence of tumor apoptosis fell clearly into two discrete groups, with small tumors exhibiting an intermediate incidence relative to angiogenic islets and large end-stage tumors (Fig. 6).

Discussion

In this study, we have examined the regulation of apoptosis and its role in the RIP-Tag mouse model of multistep carcinogenesis. Our data revealed a progressive increase in apoptosis that peaked in angiogenic islets and was subsequently reduced in two steps in solid tumors. The apoptotic process did not require the activity of the p53 tumor suppressor, a known regulator of apoptosis. Furthermore, we demonstrated functionally that reduction of apoptosis, although not affecting the preneoplastic stages, significantly enhanced tumor progression, as reflected by increased incidence of tumors developing from the angiogenic precursor stage. Finally, we presented evidence supporting a role for cell-death regulation in expansion of established tumors.

Apoptosis accompanies deregulated cell proliferation in preneoplastic stages

A number of studies have suggested that, under conditions that limit cell growth, induction of apoptosis accompanies the enhanced cell proliferation resulting from either oncogene expression or inactivation of negative cell-cycle regulators (Evan et al. 1992; Jacks et al. 1992;

Table 1. Tumor incidences of R1T2/R7bx$_L$ and R1T2 mice

Genotype	Age (weeks)	Percent of angiogenic islets that are tumors[a]	S.D.
R1T2, bcl-x_L	11	20.91	2.37
R1T2	11	8.47	5.41

Genotype	Age (weeks)	Average no. tumors/animal[a]	S.D.
R1T2, bcl-x_L	14–15	32.82	10.45
R1T2	14–15	12.38	3.46

[a]The difference in tumor incidence of R1T2/R7bx$_L$ and R1T2 mice, both at 11 and 14–15 weeks of age, is statistically significant with $P < 0.001$, as determined by the Student's t-test. The tumor incidence in 11-week-old and 14- to 15-week-old R1T2/R7bx$_L$ mice represents values which are 2.5- and 2.7-fold that of R1T2 single-transgenics, respectively.
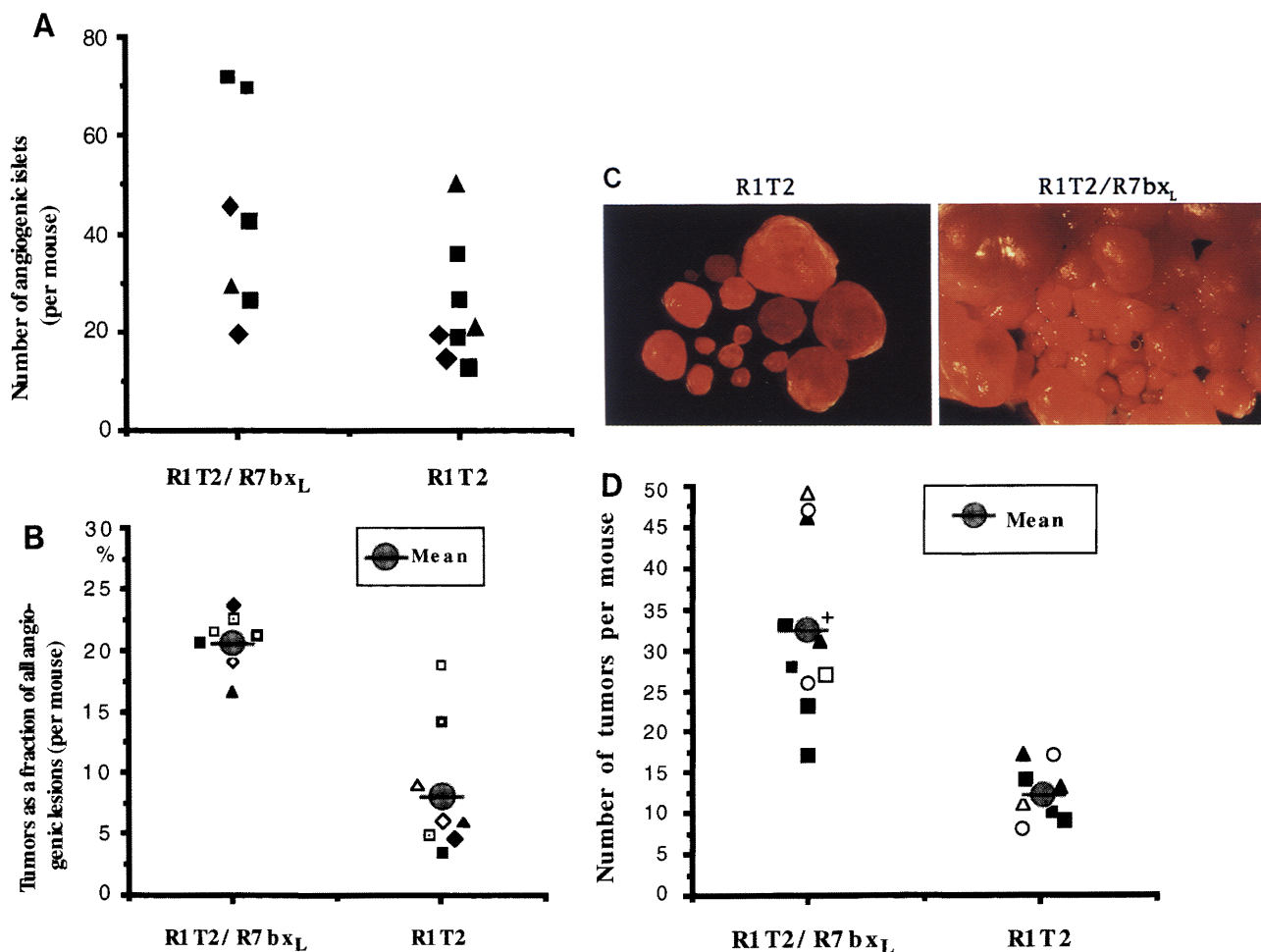
Figure 5. Analysis of angiogenic progenitor and tumor incidences in bcl-x_L overexpressing mice. (A) Angiogenic islets from 11-week-old R1T2/R7bx$_L$ and R1T2 littermates were isolated by retrograde perfusion with collagenase solution. Angiogenic islets were identified as those that exhibited a reddish patch (caused by hemorrhaging) in a white background. The incidence in individual mice is shown. (B) To determine the frequency of conversion from angiogenic progenitor islets to tumors, the number of tumors in each mouse was also determined and expressed as a percentage of the sum of angiogenic islets and tumors found in the same animal (C) Tumor incidence exemplified in two 14.5-week-old mice. Tumors isolated by microdissection from a single R1T2 (C, left) and R1T2/R7bx$_L$ (C, right) animal are shown; each red spheroid represents a single encapsulated solid tumor. Magnification, 20×. (D) The difference in tumor incidence in mice of both genotypes is reflected in the statistical determination of tumor number in these mice.

Howes et al. 1994; Morgenbesser et al. 1994; Pan and Griep 1994; White et al. 1994; White 1996). In this study, we have provided in vivo evidence correlating the onset of unscheduled oncogene-driven cell proliferation with apoptosis during the preneoplastic stages of neoplastic transformation. Despite expressing the Tag oncoprotein, normal prehyperplastic islets are quiescent, with a corresponding absence of apoptosis. Hence, expression of an oncogene itself does not appear to be sufficient to cause apoptosis if proliferation is not activated. In hyperplastic and angiogenic islets, Tag apparently induces aberrant cell proliferation under conditions that do not initially include a sufficient repertoire of confirmatory signals for that decision, of which Igf-2 represents one class (Christofori et al. 1994; Naik et al. 1994) and, herein, the bcl-2 family members describe a second. Consequently, a significant number of the cells in the early stages undergo cell death, consistent with a previous study documenting c-*myc*-induced cell proliferation and death under serum starvation in vitro (Evan et al. 1992).

The incidence of apoptosis in RIP-Tag preneoplastic stages increases with increasing cell proliferation, but is quenched in tumors. Tumors represent a dramatic increase in size (~500–1000× on average) compared with angiogenic islets, even though their mitotic index is no higher than the preceding preneoplastic stage. We conclude that the accumulation of cells comprising the tumor mass must result from a significant reduction in cell death within these tumors. Thus, both cell proliferation and death protection collaborate in the etiology of cancer.

Protection from cell death does not affect early events in tumorigenesis

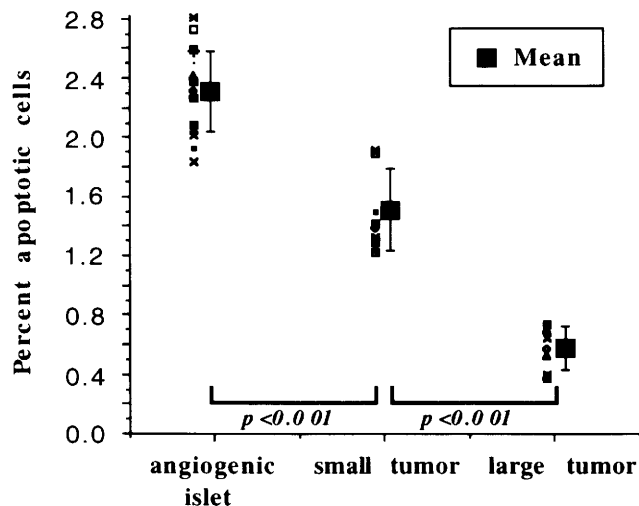Because angiogenesis in mature tumors increasingly ap-

Figure 6. Analysis of apoptotic incidence in small and large tumors. The TUNEL assay was utilized to assess the incidence of apoptosis in small and large tumors, with angiogenic islets included as a reference point. Each data point represents the index for a single islet or tumor; a total of 15 angiogenic islets, 10 small tumors, and 10 large tumors from nine mice aged 10 to 15 weeks were analyzed. The differences in apoptotic incidence between angiogenic islet and small tumor and between small and large tumor are statistically significant ($P<0.001$ in each case) by the Student's t-test.

pears to serve as a down-modulator of apoptosis (Holmgren et al. 1995; Parangi et al. 1996), the cell-death incidence we observed in angiogenic islets was unexpectedly high. This conundrum led us to ask whether one of the effects of the stepwise increases in apoptosis was to limit the number of hyperplastic islets (50% of ~400 islets) that progress to the angiogenic stage. We hypothesized that if this were true, then conferring protection to all Tag-expressing cells by coexpressing the bcl-x_L death protector would accelerate the accumulation of the angiogenic preneoplastic lesions. Our data, however, demonstrate a similar incidence of angiogenic islets in both R1T2/R7bx$_L$ and R1T2 mice, a correspondence that argues against a functional role for death-resistance in the preneoplastic stages. Thus, the high apoptotic index in angiogenic islets may be symptomatic of the microenvironment at this stage, but not a determinant of it.

Protection from apoptosis functionally contributes to formation of tumors from angiogenic progenitors

Studies in animal models (Howes et al. 1994; Pan and Griep 1994; Symonds et al. 1994) and human cancers (Bedi et al. 1993) have indicated a correlation between reduction of apoptosis and tumor formation. However, it was unclear when and where regulation of apoptosis played a role in the development of these tumors. In one pertinent study, Tag-driven brain tumors in mice developed faster when p53 was inactivated; tumor formation was correlated with lower apoptosis (Symonds et al. 1994). However, since p53 has multiple functions, in-

cluding control of cell growth and death, regulation of the mitotic checkpoint (Cross et al. 1995), and suppression of angiogenesis (Dameron et al. 1994), inactivation of such a multifunctional factor could have effects in addition to reduction of apoptosis that contributed to tumor development. Therefore, in the present study we examined the direct consequence of ablating apoptosis during multistep tumorigenesis. We genetically engineered mice to express a gene, bcl-x_L, the sole known function of whose product is conferral of protection from apoptosis (Boise et al. 1993). RIP-Tag/RIP-bcl-x_L mice exhibited a marked decrease in cell death in preneoplastic stages, to levels comparable to those normally observed in single-transgenic RIP-Tag tumors. But there was no change in the incidence or timing of the preneoplastic stages. In contrast, there was a significant ~2.5-fold increase in the cumulative incidence of solid tumors that arose out of angiogenic progenitors. This result, therefore, clearly demonstrates a functional consequence of down-regulation of apoptosis in the transition from angiogenic islets to tumors. Hence, we present direct in vivo evidence that protection from apoptosis in preneoplastic cells can enhance progression to neoplasia.

Apoptosis is a likely modulator of tumor size

Several lines of evidence support the conclusion that protection from apoptosis contributes to subsequent growth of islet cell carcinomas, following their progression from the angiogenic progenitor stage. First, in this study we showed that tumor size correlates closely with reduced apoptosis. Moreover, the incidence of apoptosis in small and large tumors fell into two clusters, indicating discrete steps in the transition from the high apoptosis characteristic of angiogenic islets to the low level in large end-stage tumors; small nascent tumors were found to exhibit a discrete intermediate apoptotic index. This two-step feature identifies two stages of progression within the classification of solid tumors and suggests that the transition from a small to a large tumor requires a specific change that leads to further protection from death; reduced apoptosis is not simply an effect of tumor growth, but rather is an actively regulated process that causally contributes to it. The second line of evidence comes from R1T2 mice that lack IGF-2, a cytokine that confers survival function (Barres et al. 1992; Harrington et al. 1994). R1T2/Igf-2-null mice developed tumors that were markedly reduced in size, with the same high mitotic index as those from wild-type animals, but exhibiting a striking increase in apoptosis (Christofori et al. 1994; Naik et al. 1994). Third, in R1T2 mice treated with angiogenesis inhibitors, vessel density was halved and tumors were also dramatically reduced in size (Parangi et al. 1996). Again, although the proliferation index was unchanged relative to untreated animals, there was a significant increase in apoptosis. Taken together, these observations establish a clear correlation between tumor growth and levels of cell death, and thus provide strong support for regulation of tumor size by apoptosis.

Apoptosis in RIP-Tag islet cells is not p53 dependent

Previous studies have clearly established the requirement for p53 function in oncogene-induced apoptosis of a number of cell types (Debbas and White 1993; Hermeking and Eick 1994; Howes et al. 1994; Pan and Griep 1994; Symonds et al. 1994; Wagner et al. 1994). In the present study, however, we have determined that p53 expression is not required for apoptosis in Tag-expressing islet cells. The observation that RIP-Tag/p53$^{-/-}$ mice have a similar apoptotic phenotype to single-transgenic RIP-Tag mice suggests that Tag blocks any apoptosis-inducing function of p53 in these cells. Moreover, even if p53 can contribute to apoptosis in this cell type, it is clearly not essential, given the dynamic regulation of apoptosis seen in both types of mice.

Although p53-dependent death is clearly an important component of the apoptotic response of many cell types to a variety of stimuli (Clarke et al. 1993; Lowe et al. 1993a,b), until now, the importance of p53-independent apoptosis in cancer was not apparent. One recent study suggested that p53 loss actually sensitizes some cells to taxol-induced apoptosis (Wahl et al. 1996). Beyond that specific clue, the fact that p53-null mice develop normally (Donehower et al. 1992) argues that the p53-independent pathway is an important component of developing tissues' capability to execute apoptosis. In this study we have extensively characterized a pattern of p53-independent apoptosis that is dynamically regulated in a multistep tumorigenesis pathway. Moreover, we present evidence correlating initiation of deregulated cell proliferation with induction of p53-independent cell death, the abrogation of which resulted in a significant enhancement of tumor development.

Multiple regulators of cell death in RIP-Tag islet cells

Collectively we have identified both intracellular and transcellular regulators of apoptosis in RIP-Tag islets (Fig. 7). In the present study, we showed that both positive (bcl-x_L and bag-1) and negative (bax) intracellular regulators are expressed at significant levels in the RIP-Tag islets (Figs. 3, and 7A). One can postulate that if functional p53 was present in the islet cells, it too could potentially modulate apoptosis in these cells as an intracellular regulator (Fig. 7B). Regarding transcellular regulators, we have shown previously that the secreted protein Igf-2 serves as a down-modulator of apoptosis in this pathway (Christofori et al. 1994). In addition, two recent studies have implicated angiogenesis as a paracrine regulator of apoptosis in tumors, both in this model (Parangi et al. 1996) and in a classical transplantation model of Lewis lung carcinoma (Holmgren et al. 1995).

The presence of Igf-2 and angiogenesis, while demonstrably important for maintaining low cell death in established tumors, apparently cannot account for the down-regulation of apoptosis observed in the transition from angiogenic islets to tumors, as each parameter has similar characteristics in both stages (Fig. 7A). Two intracellular regulators, bag-1 and bax, are also expressed at
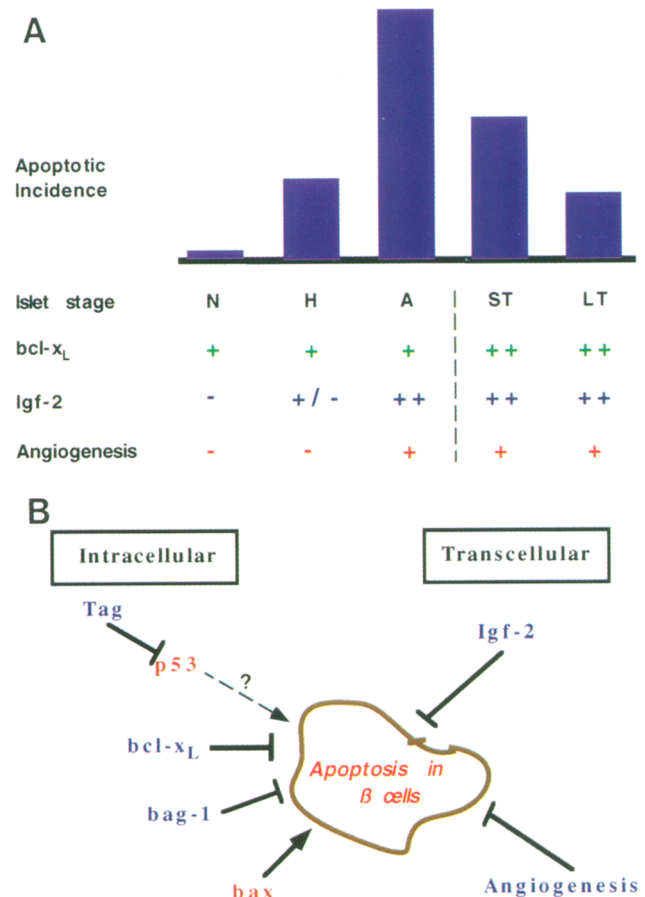

Figure 7. Summary of apoptosis regulatory factors expressed in RIP-Tag multistep tumorigenesis. (*A*) A number of protective regulators of apoptosis are differentially expressed during the multistep progression of RIP-Tag tumorigenesis. The increase in bcl-x_L expression coincides with the reduction in apoptosis observed in the transition from angiogenic progenitor islets to tumors. Igf-2 expression and angiogenesis, on the other hand, are induced in angiogenic islet progenitors and remain relatively unchanged in tumors. Note, however, that neovascularization of angiogenic islets and tumors may differ qualitatively; it is possible that new blood capillaries in angiogenic islets are less developed and more prone to hemorrhage compared with those found in established tumors. (N) normal Tag$^+$ islets; (H) hyperproliferative islets (carcinoma in situ); (A) angiogenic islets; (ST) small nascent tumor; and (LT) large end-stage tumor. (*B*) The survival or death of oncogene-expressing islet β-cells is likely to be determined through the integration of signals from multiple intra- and transcellular apoptosis regulatory factors. In addition, it is possible that p53, if it were not inactivated by the Tag oncogene, would also be an effector of apoptosis.

similar levels in these stages. Therefore, it is reasonable to conclude from the functional tests afforded by the forced overexpression of bcl-x_L in double transgenic mice that the observed up-regulation of endogenous bcl-x_L in the standard (single transgenic) tumorigenesis pathway contributes to the significant reduction in apoptosis that is evident in the solid tumors. We suspect that an

additional, as-yet-undefined modulator of apoptosis is also involved, for the following reasons: (1) transgene-driven overexpression of bcl-x_L does not completely abrogate apoptosis in Tag oncogene-expressing cells (Fig. 4), and (2) there is no obvious difference in the levels of Igf-2, bcl-x_L, or angiogenesis when small tumors having intermediate apoptosis are compared with large tumors showing low apoptosis (Fig. 7A).

The observation that these diverse apoptotic regulatory factors are each functionally involved in controlling cell-death decisions in islet β cells is indicative of a mechanism that integrates signals from both trans- and intracellular factors (Fig. 7B). The existence of multiple apoptosis regulatory signals in this one cell type may have important implications for the prevention or treatment of cancers; distinct components of the apoptotic pathway could potentially be targeted simultaneously. Moreover, the evidence that these signals can each regulate apoptosis in the absence of wild-type p53 function engenders optimism for the development of effective treatment strategies for the ~50% of human cancers in which p53 is either mutated or absent, particularly because we have demonstated in two previous studies (Christofori et al. 1994; Parangi et al. 1996) that the growth of established islet cell tumors with abrogated p53 (via the Tag oncogene) can be restrained by increasing the incidence of apoptosis. In that light, it is notable that many cancers have been shown to have elevated expression of either bcl-2 or its functional homolog bcl-x_L (McDonnell et al. 1992, 1993; Schott et al. 1995; Thompson 1995), and either Igf-2 or its family members (Osborne et al. 1989; Yee et al. 1991). Hence, these classes of death protectors could well present attractive targets for the development of cancer therapies that antagonize their functions.

Materials and methods

Tissue preparation

For the TUNEL assay, mice were perfusion fixed with 4% paraformaldehyde, and then the pancreas was removed and postfixed in 4% paraformaldehyde overnight. Tissues were then dehydrated through 50%, 70%, 80%, 95%, and 100% ethanol and xylene, followed by embedding in paraffin (Paraplast). Sections of 5-μm thickness were used for staining. For BrdU staining, tissues were prepared as above, except that mice were injected intraperitoneally with 100 μg of BrdU per gram body weight 2–3 hr prior to sacrifice. For in situ hybridization, mice were also perfusion fixed with 4% paraformaldehyde, followed by removal of pancreas and an overnight postfixation in 4% paraformaldehyde. Tissues were then immersed in 30% sucrose for about 6 hr before being embedded in OCT compound (Tissue Tek). Cryosections of 10-μm thickness were used for in situ hybridization.

TUNEL assay

Staining for apoptotic cells was performed essentially as described previously (Surh and Sprent 1994) with some modifications. Paraffin tissue sections were deparaffinized in xylene, rehydrated through an alcohol series, and then pretreated in pro-

teinase K (1–2 μg/ml) for 10 min at room temperature. Gibco-BRL TdT reaction buffer supplemented with 0.15 M NaCl and 0.05% bovine serum albumin (BSA) was used. Digoxigenin-labeled dUTP (Boehringer Mannheim) was used at a concentration of 3 mM. TdT reaction conditions were 200 U/ml enzyme (Gibco BRL) for 30 min at 37°C, followed by incubation with peroxidase-labeled anti-digoxigenin antibody (Boehringer Mannheim) at a concentration of 5 U/ml for 30 min at room temperature. Labeled cells were detected by treatment with diaminobenzidine and H_2O_2, with a reaction time of ~4 min.

In situ hybridization

In situ hybridization was performed as described previously (Naik et al. 1994). The bcl-x_L probe comprised a PCR fragment of 765 bp amplified with specific primers for murine bcl-x (5′ primer: 5′-TTGGACAATGGACTGGTTGA-3′; 3′ primer: 5′-GTAGAGTGGATGGTCAGTG-3′), and the bax probe consisted of a 492-bp PCR fragment amplified with primers specific for murine bax (5′ primer: 5′-TTTCATCCAG-3′; 3′ primer: 5′-TCAGCCCATCTTCTTCCAGATG-3′); cDNA from a β tumor cell line, βTC-3, was used. Cloning into plasmid pAMP19 was done by use of the UDG cloning system (Gibco BRL), according to the manufacturer's instructions. Following this, SalI–SpeI fragments from the pAMP19 plasmids containing the respective PCR fragments were subcloned into pBluescript KS⁻ between SalI and SpeI. Antisense riboprobe synthesis was performed with T7 polymerase on plasmids linearized with SalI. The bag-1 probe comprised a 659-bp PCR fragment amplified from mouse spleen cDNA with murine bag-1-specific primers (5′ primer: 5′-ATGGCCAAGACCGAGGAGAT-3′ and 3′ primer: 5′-TCATTCAGCCAGGGCCAAGT-3′). The PCR fragment was end filled and subcloned into the EcoRV site of pBluescript KS⁻. For synthesis of probes, the plasmid was linearized with SpeI and T3 polymerase was used.

BrdU immunohistochemistry

Tissues prepared for BrdU staining, as described above, were immunostained as described previously (Naik et al. 1994), with some minor modifications. Tissues were treated with Protease Type XXIV (Sigma) instead of proteinase K for 30 sec. Reaction time for diaminobenzidine treatment was 4 min.

Generation of R1T2, p53-null mice

R1T2 mice of C57/Bl6 strain were first crossed with p53-null mice of FVB/n background. F_1 R1T2/p53$^{+/−}$ and p53$^{+/−}$ animals were intercrossed to generate R1T2/p53$^{−/−}$ and wild-type R1T2 mice. We have determined previously that apoptosis in islet cells is not affected by this mixed genetic background.

Generation and breeding of RIP7-bcl-x_L mice

A 0.8-kb EcoRI fragment of human bcl-x_L was released from pSFFV-bcl-x_L (courtesy of Dr. Craig Thompson, Univ. of Chicago) and blunt-end ligated into the RIP7 vector at the ClaI site; the RIP7 vector consists of a 9.5-kb 5′ regulatory region for the rat insulin II gene, the first intron of the insulin gene, and a pBluescript backbone. The transgene DNA was linearized at the SalI site, purified by CsCl banding and prepared for injection into fertilized one-cell embyos derived from matings of FVB/n males and females. Injection of transgene DNA was performed as described previously (Hanahan 1985). Two independent founder lines were obtained. These lines were subsequently backcrossed into C57/Bl6 for at least two generations; most

animals analyzed were backcrossed for three to four generations.

Assessment of angiogenic islet and tumor incidence

Angiogenic islets and tumors from 11-week-old mice were isolated by retrograde perfusion with collagenase solution through the common bile duct (Lacy and Kostianovsky 1967). Angiogenic islets were identified as islets with a reddish patch on a whitish background, and tumors were identified as solid encapsulated large islet structures with an overall red hue. In the analysis of 14- to 15-week-old mice, animals were maintained on a high-carbohydrate diet; mice were fed ad libitum with 10% sucrose water and a high-carbohydrate solid food (Teklad Diet). This diet enabled us to partially ameliorate deaths from insulinoma-induced hypoglycemia in mice that bore high tumor burden. Indeed, in initial determinations of tumor incidence, our data were obscured by the selective deaths of these mice. Tumors from 14- to 15-week-old mice were isolated by microdissection. In some cases, tumors were processed for paraffin embedding, as described above, and subsequently immunostained for insulin.

Acknowledgments

References

Adams, J.M., A.W. Harris, C.A. Pinkert, L.M. Corcoran, W.S. Alexander, S. Cory, R.D. Palmiter, and R.L. Brinster. 1985. The c-myc oncogene driven by immunoglobulin enhances induce lymphoid malignancy in transgenic mice. *Nature* **318:** 533–538.

Alpert, S., D. Hanahan, and G. Teitelman. 1988. Hybrid insulin genes reveal a developmental lineage for pancreatic endocrine cells and imply a relationship with neurons. *Cell* **53:** 295–308.

Bakhshi, A., J.P. Jensen, P. Goldman, J.J. Wright, C.W. McBride, A.L. Epstein, and S.J. Korsmeyer. 1985. Cloning the chromosomal breakpoint of t(14;18) human lymphomas clustering around JH on chromosome 14 and near a transcriptional unit on 18. *Cell* **41:** 899–906.

Barres, B.A., I.K. Hart, H.S. Coles, J.F. Burne, J.T. Voyvodic, W.D. Richardson, and M.C. Raff. 1992. Cell death and control of cell survival in the oligodendrocyte lineage. *Cell* **70:** 31–46.

Bedi, A., P.J. Pasricha, A.J. Akhtar, J.P. Barber, G.C. Bedi, F.M. Giardiello, B.A. Zehnbauer, S.R. Hamilton, and R.J. Jones. 1993. Inhibition of apoptosis during development of colorectal cancer. *Cancer Res.* **55:** 1811–1816.

Berges, R.R., Y. Furuya, L. Remington, H.F. English, and T.

Jacks. 1993. Cell proliferation, DNA repair, and p53 function are not required for programmed death of prostatic glandular cells induced by androgen ablation. *Proc. Natl. Acad. Sci.* **90:** 8910–8914.

Boise, L.H., M. Gonzalez-Garcia, C. Postema, L. Ding, T. Lindsten, L.A. Turka, X. Mao, G. Nunez, and C.B. Thompson. 1993. bcl-x, a bcl-2-related gene that functions as a dominant regulator of apoptotic cell death. *Cell* **74:** 597–608.

Christofori, G., P. Naik, and D. Hanahan. 1994. A second signal supplied by insulin-like growth factor II in oncogene-induced tumorigenesis. *Nature* **369:** 414–418.

Clarke, A.R., C.A. Purdie, D.J. Harrison, R.G. Morris, C.C. Bird, M.L. Hooper, and A.H. Wyllie. 1993. Thymocyte apoptosis induced by p53-dependent and independent pathways. *Nature* **362:** 849–852.

Cleary, M.L. and J. Sklar. 1985. Nucleotide sequence of a t(14;18) chromosomal breakpoint in follicular lymphoma and demonstration of a breakpoint cluster region near a transcriptionally active locus on chromosome 18. *Proc. Natl. Acad. Sci.* **82:** 7439–7443.

Cross, S.M., C.A. Sanchez, C.A. Morgan, M.K. Schimke, S. Ramel, R.L. Idzerda, W.H. Raskind, and B.J. Reid. 1995. A p53-dependent mouse spindle checkpoint. *Science* **267:** 1353–1356.

Dameron, K.M., O.V. Volpert, M.A. Tainsky, and N. Bouck. 1994. Control of angiogenesis in fibroblasts by p53 regulation of thrombospondin-1. *Science* **265:** 1582–1584.

Debbas, M. and E. White. 1993. Wild-type p53 mediates apoptosis by E1A, which is inhibited by E1B. *Genes & Dev.* **7:** 546–554.

Donehower, L.A., M. Harvey, B.L. Slagle, M.J. McArthur, CA. Montgomery Jr., J.S. Butel, and A. Bradley. 1992. Mice deficient of p53 are developmentally normal but susceptible to spontaneous tumours. *Nature* **356:** 215–221.

Efrat, S., S. Baekkeskov, D. Lane, and D. Hanahan. 1987. Coordinate expression of the endogenous p53 gene in beta cells of transgenic mice expressing hybrid insulin-SV40 T antigen genes. *EMBO J.* **6:** 2699–2704.

Evan, G.I., A.H. Wyllie, C.S. Gilbert, T.D. Littlewood, H. Land, M. Brooks, C.M. Waters, L.Z. Penn, and D.C. Hancock. 1992. Induction of apoptosis in fibroblasts by c-myc protein. *Cell* **69:** 119–128.

Folkman, J., K. Watson, D. Ingber, and D. Hanahan. 1989. Induction of angiogenesis during the transition from hyperplasia to neoplasia. *Nature* **339:** 58–61.

Hanahan, D. 1985. Heritable formation of pancreatic beta-cell tumours in transgenic mice expressing recombinant insulin/simian virus 40 oncogenes. *Nature* **315:** 115–122.

Harrington, E.A., M.R. Bennett, A. Fanidi, and G.I. Evan. 1994. c-Myc-induced apoptosis in fibroblasts is inhibited by specific cytokines. *EMBO J.* **13:** 3286–3295.

Hermeking, H. and D. Eick. 1994. Mediation of c-Myc-induced apoptosis by p53. *Science* **265:** 2091–2093.

Hockenberry, D., G. Nunez, C. Milliman, R.D. Schreiber, and S.J. Korsmeyer. 1990. Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. *Nature* **348:** 334–336.

Holmgren, L., M.S. O'Reilly, and J. Folkman. 1995. Dormancy of micrometastases: Balanced proliferation and apoptosis in the presence of angiogenesis suppression. *Nature Med.* **1:** 149–153.

Howes, K.A., N. Ransom, D.S. Papermaster, J.G.H. Lasudry, D.M. Albert, and J.J. Windle. 1994. Apoptosis or retinoblastoma: Alternative fates of photoreceptors expressing the HPV-16 E7 gene in the presence or absence of p53. *Genes & Dev.* **8:** 1300–1310.

Jacks, T., A. Fazeli, E.M. Schmitt, R.T. Bronson, M.A. Goodell, and R.A. Weinberg. 1992. Effects of an Rb mutation in the mouse. *Nature* **359:** 295–300.

Kerr, J.F.R., J. Searle, B.V. Harmon, and C.J. Bishop. 1987. Apoptosis. In *Perspectives on mammalian cell death* (ed. C.S. Potten), pp. 93–128. Oxford University Press, Oxford, UK.

Korsmeyer, S.J. 1992. Bcl-2 initiates a new category of oncogenes: Regulators of cell death. *Blood* **80:** 879–886.

Lacy, P.E. and M. Kostianovsky. 1967. Method for the isolation of intact islets of Langerhans from the rat pancreas. *Diabetes* **16:** 35–39.

Lowe, S.W., H.E. Ruley, T. Jacks, and D.E. Housman. 1993a. p53-Dependent apoptosis modulates the cytotoxicity of anticancer agents. *Cell* **74:** 957–967.

Lowe, S.W., E.M. Schmitt, S.W. Smith, B.A. Osborne, and T. Jacks. 1993b. p53 is required for radiation-induced apoptosis in mouse thymocytes. *Nature* **362:** 847–849.

Ludlow, J.W. 1993. Interactions between SV40 large-tumor antigen and the growth suppressor proteins pRB and p53. *FASEB J.* **7:** 866–871.

McDonnell, T.J. and S.J. Korsmeyer. 1991. Progression from lymphoid hyperplasia to high-grade malignant lymphoma in mice transgenic for the t(14;18). *Nature* **349:** 254–256.

McDonnell, T.J., P. Troncoso, S.M. Brisbay, C. Logothetis, L.W.K. Chung, J. Hsieh, S. Tu, and M.L. Campbell. 1992. Expression of the protooncogene bcl-2 in the prostate and its association with emergence of androgen-independent prostate cancer. *Cancer Res.* **52:** 6940–6944.

McDonnell, T.J., M.C. Marin, B. Hsu, S.M. Brisbay, K. McConnell, S.M. Tu, M.L. Campbell, and J. Rodriguez-Villanueva. 1993. The bcl-2 oncogene: Apoptosis and neoplasia. *Radiat. Res.* **136:** 307–312.

Morgenbesser, S.D., B.O. Williams, T. Jacks, and R.A. DePinho. 1994. p53-Dependent apoptosis produced by Rb-deficiency in the developing mouse lens. *Nature* **371:** 72–74.

Motoyama, N., F. Wang, K.A. Roth, H. Sawa, K. Nakayama, I. Negishi, S. Senju, Q. Zhang, S. Fujii, and D.Y. Loh. 1995. Massive cell death of immature hematopoietic cells and neurons in Bcl-x-deficient mice. *Science* **267:** 1506–1510.

Naik, P., G. Christofori, and D. Hanahan. 1994. Insulin-like growth factor II is focally up-regulated and functionally involved as a second signal for oncogene-induced tumorigenesis. *Cold Spring Harbor Symp. Quant. Biol.* **59:** 459–470.

Oltvai, Z.N., C.L. Milliman, and S. Korsmeyer. 1993. Bcl-2 heterodimerizes in vivo with a conserved homolog, bax, that accelerates programmed cell death. *Cell* **74:** 609–619.

Oppenheim, R.W. 1991. Cell death during development of the nervous system. *Annu. Rev. Neurosci.* **14:** 453–501.

Osborne, C.K., E.B. Coronado, L.J. Kitten, C.I. Arteaga, S.A. Fuqua, and K. Ramaharma. 1989. Insulin-like growth factor-II: A potential autocrine/paracrine growth factor for human breast cancer acting via the IGF-I receptor. *Mol. Endocrinol.* **3:** 1701–1709.

Pan, H. and A.E. Griep. 1994. Altered cell cycle regulation in the lens of HPV-16 E6 or E7 transgenic mice: Implications for tumor suppressor gene function development. *Genes & Dev.* **8:** 1285–1299.

Parangi, S., W. Dietrich, G. Christofori, E.S. Lander, and D. Hanahan. 1995. Tumor suppressor loci on mouse chromosomes 9 and 16 are lost at distinct stages of tumorigenesis in a transgenic model of islet cell carcinoma. *Cancer Res.* **55:** 6071–6076.

Parangi, S., M. O'Reilly, G. Christofori, L. Holmgren, J. Grosfeld, J. Folkman, and D. Hanahan. 1996. Antiangiogenic therapy of transgenic mice impairs de novo tumor growth.

Proc. Natl. Acad. Sci. **93:** 2002–2007.

Raff, M. 1992. Social controls on cell survival and cell death. *Nature* **356:** 397–400.

Schott, A.F., I.J. Apel, G. Nunez, and M.F. Clarke. 1995. Bcl-XL protects cancer cells from p53-mediated apoptosis. *Oncogene* **11:** 1389–1394.

Strasser, A., A.W. Harris, M.L. Bath, and S. Cory. 1990. Novel primitive lymphoid tumours induced in transgenic mice by cooperation between myc and bcl-2. *Nature* **348:** 331–333.

Strasser, A., A.W. Harris, T. Jacks, and S. Cory. 1994. DNA damage can induce apoptosis in proliferating lymphoid cells via p53-independent mechanisms inhibitable by bcl-2. *Cell* **79:** 329–339.

Surh, C.D. and J. Sprent. 1994. T-cell apoptosis detected in situ during positive and negative selection in the thymus. *Nature* **372:** 100–103.

Symonds, H., L. Krall, L. Remington, M. Aenz-Robles, S. Lowe, T. Jacks, and T. Van Dyke. 1994. p53-Dependent apoptosis suppresses tumor growth and progression in vivo. *Cell* **78:** 703–711.

Takayama, S., T. Sato, S. Krajewski, K. Kochel, S. Irie, J.A. Millan, and J.C. Reed. 1995. Cloning and functional analysis of BAG-1: A novel Bcl-2-binding protein with anti-cell death activity. *Cell* **80:** 279–284.

Teitelman, G., S. Alpert, and D. Hanahan. 1988. Proliferation, senescence, and neoplastic progression of beta cells in hyperplasic pancreatic islets. *Cell* **52:** 97–105.

Thompson, C.B. 1995. Apoptosis in the pathogenesis and treatment of disease. *Science* **267:** 1456–1462.

Tsujimoto, Y., J. Gorham, J. Cossman, E. Jaffe, and C.M. Croce. 1985. The t(14;18) chromosome translocations involved in B-cell neoplasms result from mistakes in VDJ joining. *Science* **229:** 1390–1393.

Vaux, D.L., S. Cory, and T.M. Adams. 1988. Bcl-2 promotes the survival of hematopoietic cells and cooperates with c-myc to immortalize pre-B cells. *Nature* **335:** 440–442.

Wagner, A.J., J.M. Kokontis, and N. Hay. 1994. Myc-mediated apoptosis requires wild-type p53 in a manner independent of cell cycle arrest and the ability of p53 to induce $p21^{wafl/cip1}$. *Genes & Dev.* **8:** 2817–2830.

Wahl, A.F., K.L. Donaldson, C. Fairchild, R.Y.F. Lee, S.A. Foster, G.W. Demers, and D.A. Galloway. 1996. Loss of normal p53 function confers sensitization to Taxol by increasing G2/M arrest and apoptosis. *Nature Med.* **2:** 72–79.

White, E. 1996. Life, death, and the pursuit of apoptosis. *Genes & Dev.* **10:** 1–15.

White, E., L. Rao, S.K. Chiou, C.C. Tseng, P. Sabbatini, M. Gonzalez, and P. Verwaerde. 1994. Regulation of apoptosis by the transforming gene products of adenovirus. In *Apoptosis* (ed. E. Mihich and R.T. Schimke), pp. 47. Plenum Press, New York, NY.

Wyllie, A.H. 1980. Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. *Nature* **284:** 555–556.

Yee, D., N. Rosen, R.E. Favoni, and K.J. Cullen. 1991. The insulin-like growth factors, their receptors, and their binding proteins in human breast cancer. *Cancer Treat. Res.* **53:** 93–106.

Ziegler, A., A.S. Jonason, D.J. Leffell, J.A. Simon, H.W. Sharma, J. Kimmelman, L. Remington, T. Jacks, and D.E. Brash. 1994. Sunburn and p53 in the onset of skin cancer. *Nature* **372:** 773–776.

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