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RAPID COMMUNICATION

Inhibition of HIV-1 Tat Activity Correlates with Down-Regulation of *bcl*-2 and Results in Reduction of Angiogenesis and Oncogenicity

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The Tat protein of the human immunodeficiency virus type 1 promotes survival and growth and inhibits apoptosis of different cell types. These effects of Tat are attributed to the induction of *bcl*-2 gene expression. In this study we show that the blocking of both intracellular and extracellular Tat correlates with a decrease of *bcl*-2 transcripts, leading *in vitro* to a lower growth rate and attenuation of the transformed phenotype and *in vivo* to a reduced angiogenic and oncogenic activity of Tat-expressing cells. These results support the notion that *bcl*-2 is an effector of Tat-induced angiogenesis and oncogenesis and indicate that the blocking of Tat functions by immunoprophylactic, pharmacological, and gene therapy approaches may help to control oncogenesis during AIDS.

Key Words: HIV-1 Tat protein; *bcl*-2 expression; quantitative RT-PCR; apoptosis; angiogenesis; oncogenicity.

Introduction. The Tat protein of the human immunodeficiency virus type 1 (HIV-1) is an early gene product essential for virus gene expression and replication (*2, 15*). Tat promotes transcription of the viral genome by interacting with the *trans*-activation responsive (TAR) element, located at the 5′ end of all viral mRNAs (*17, 26*). Tat is released into the culture medium in a biologically active form by either HIV-1-infected or *tat*-transfected cells in the absence of cell death (*7, 14*) and displays pleiotropic activities on survival and growth of different cell types (*5, 13, 21, 33*). In extracellular form and as a recombinant molecule, Tat shows angiogenic activity and stimulates adhesion, migration, and invasion of spindle-shaped cells derived from AIDS Kaposi's sarcoma (KS) lesions and of normal endothelial cells activated by inflammatory cytokines (*1, 3*). In addition, extracellular Tat behaves as an autocrine and paracrine growth factor protecting epithelial, lymphoid, and neuronal cells from apoptosis induced by serum starvation (*5, 33*). The effects of Tat on cell survival and growth have been correlated with an increased expression of cellular genes encoding transforming growth factor-β1, tumor necrosis factor-β, interleukin-2, and interleukin-6 (*4, 27, 31, 32*) or with modulation of expression of cellular genes involved in the control of the cell cycle and apoptosis such as *p*53 (*19*) and *bcl*-2 genes (*30, 34, 35*).

bcl-2 belongs to a gene family encoding proteins that inhibit (*bcl*-2, Bcl-xL, Bcl-w, Bfl-1, and Mcl-1) or promote (Bax, Bad, Bak, Bcl-X, Bid, and Hrk) apoptosis (*18*). Tat upregulates *bcl*-2 gene expression both in Jurkat cells and in PBMC (*34*). In addition, it was shown that Tat activates *bcl*-2 promoter-directed gene expression and that either N- or C-terminal sequences of Tat are required for optimal transactivation (*30*). In the present study we have analyzed whether inhibition of intracellular and extracellular Tat induces down-modulation of *bcl*-2 gene expression and consequently reduction or inhibition of the oncogenic and angiogenic effects of Tat.

Results and Discussion: Generation of cells expressing the antisense tat gene. The aim of this study is to determine whether the blocking of Tat activity reduces or inhibits the oncogenic and angiogenic effects of cells expressing Tat. T53 cells are derived from an adenocarcinoma of a *tat* transgenic mouse and constitutively express and release Tat protein in the culture medium (*10, 11*). T206 cells, which are also derived from an adenocarcinoma of a *tat* transgenic mouse and harbor the *tat* gene, are negative for Tat expression (*11*) (Table 1). T53 and T206 cells were transfected with a plasmid contain-

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TABLE 1

Quantitative Determination of Tat Protein

Cell lines	Tat molecules per cell
T53	2200
T53AScl2	700
T53AScl3	600
T53AScl4	400
T53AScl6	550
T206	0
T206AScl2	0
pRPc[a]	0

Note. Cells were analyzed by flow cytometry and the quantitative analysis was carried out as described under Materials and Methods.

[a] Mouse cells immortalized by BK virus early region, not harboring the *tat* gene and used as negative control.

ing the full-length HIV-1 *tat* cDNA in antisense orientation (*tat*AS) and the *neo* gene as the selectable marker. Several clones, obtained from T53 and T206 cell cultures, were selected and propagated in a culture medium containing geneticin (200 μg/ml). Three of these clones (T53AScl2, T53AScl4, and T206AScl2) were studied in detail. The Tat protein was detected in cells by flow cytometry (Fig. 1). Quantitative analysis indicated that the mean number of Tat molecules per cell was 2200 in T53 cells, 700 in T53AScl2, 600 in T53AScl3, 400 in T53AScl4, and 550 in T53AScl6 cells (Table 1). Since similar values were observed in six other T53AS clones, where the number of Tat molecules ranged between 400 and 700 per cell, these results indicate that transfection of a *tat* cDNA in antisense orientation in T53 cells consistently reduced, but did not completely abrogate, Tat expression. Indeed, T53AScl2 cells were able to survive without signs of apoptosis in serum-free medium like T53 cells (Figs. 2A and 2D), even though at a lower growth rate (doubling time 53 h) compared to T53 cells (doubling time 32 h), suggesting that Tat is still released in the culture medium in amounts sufficient to inhibit apoptosis. In fact, an identical apoptotic process was triggered in T53AScl2 and T53 cells (Figs. 2B, 2C, 2E, and 2F), when they were treated with an anti-Tat antibody or with PNU153429, a distamycin A derivative which binds extracellular Tat and neutralizes its functions (*12*). We also evaluated the apoptotic death by flow cytometry. Treatment with an anti-Tat polyclonal antibody induced apoptosis, indicated by the appearance of a subdiploid peak, in 37.0% of T53 cells and 27.5% of T53AScl2 cells, compared to a value of 7.5% ($P < 0.00001$) and 10.0% ($P < 0.00001$), respectively, in untreated cells (Figs. 3A and 3B).

We then analyzed the *in vitro* growth characteristics of T53AScl2 and T53AScl4 cells. These cells display lower saturation density and clonogenic activity on plastic and higher serum dependence than T53 cells (data not shown). In addition, T53AScl2 cells produced significantly lower amounts of urokinase-type plasminogen activator (3.2 mU/μg protein) than T53 cells (11 mU/μg protein). These results indicate that the block of Tat correlates with a reduced transformation phenotype *in vitro*.

Determination of oncogenicity and angiogenesis. To assay whether inhibition of Tat expression reduces oncogenicity, T53AScl2 and T53AScl4 cells were inoculated subcutaneously in nude mice. T53 cells were included as a control. As shown in Table 2, all animals developed tumors with a short latency period (2–6 days). Thirty days after cell inoculation, mice were sacrificed to measure the tumor volume and to determine the presence of metastases. This analysis indicated that the volume of tumors induced by T53AScl2 and T53AScl4 cells was significantly smaller than that of tumors induced by T53 cells ($P < 0.001$ and $P < 0.01$, respectively). Moreover, four of six animals inoculated with T53 cells developed a total of 33 metastases in lungs, lymph nodes, kidneys, and heart, whereas T53AScl2 and T53AScl4 cell clones produced 2 and 11 metastases, respectively, in two of four animals ($P < 0.0005$ and $P < 0.05$). The extent of neoangiogenesis in tumors was analyzed in a separate experiment as described under Materials and Methods. The results indicate that the number of capillaries was significantly lower in tumors induced by T53AScl2 and T53AScl4 cells than in tumors induced by T53 cells ($P < 0.001$) (Table 2). Moreover, histologic analysis of tumors induced by T53AScl2 cells showed the presence of several necrotic areas which were absent in tumors induced by T53 cells. These data indicate that the inhibition of Tat expression reduces tumorigenicity and angiogenic activity of T53AS clones, thus leading to a decreased growth and metastatic dissemination of tumors.

Quantitative analysis of bcl-2 transcripts. Since several data indicate that Tat protects cells from apoptosis through activation of *bcl-2* gene expression (*30, 34*) and

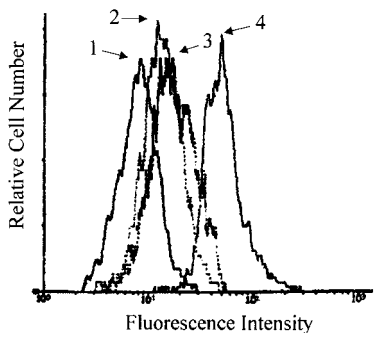

FIG. 1. Detection of Tat protein by flow cytometry. Exponentially growing cells were analyzed as described under Materials and Methods. (1) Negative control represented by T53 cells treated with a nonspecific primary mouse monoclonal antibody; (2) T53AScl4 cells; (3) T53AScl2 cells; (4) T53 cells. Samples 2, 3, and 4 were treated with a primary mouse anti-Tat monoclonal antibody.

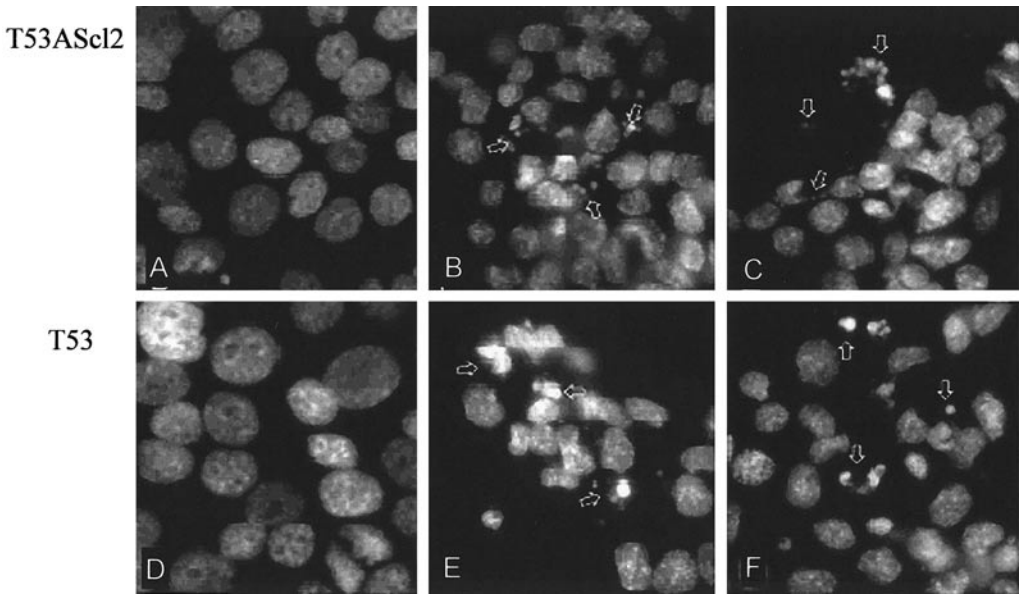

FIG. 2. Analysis of apoptosis in T53AScl2 and T53 cells maintained in culture for 48 h in serum-free medium. T53AScl2 and T53 cells were untreated (A and D) and treated with a rabbit anti-Tat polyclonal antibody (B and E) or PNU153429 (C and F). DAPI staining was carried out as described under Materials and Methods. Arrows mark apoptotic cells and micronuclei. Magnification, 75×.

that *bcl*-2 is oncogenic (*29*) and angiogenic *in vitro* and *in vivo* (*23, 24*), we determined whether inhibition of Tat correlates with down-regulation of *bcl*-2. To this purpose a quantitative reverse transcription polymerase chain reaction (qRT-PCR) was set up as described under Materials and Methods. The level of *bcl*-2 mRNA was measured in T53AScl2 and T53AScl4 cells at different time intervals (12, 24, 48, and 72 h) in the presence or absence of serum and compared to *bcl*-2 expression in T53 cells. Equal amounts of mouse wild-type (wt) *bcl*-2 RNA, equivalent to 10^5 cells, were retrotranscribed and coamplified together with 10,000, 5000, 2500, 1250, 625, and 312 molecules of a deleted *bcl*-2 RNA competitor. The PCR products were analyzed on a polyacrylamide gel to separate the 95-bp wt from the 83-bp deleted *bcl*-2 fragment (Fig. 4A, lanes 1 to 6). The results of these experiments showed that after 48 h of culture in DMEM-F12 medium with 10% fetal bovine serum (FBS), T53AScl2 cells expressed 2170 copies of *bcl*-2 transcripts per 10^5 cells, a value 2.5-fold lower than the number of RNA copies (5457) detected in T53 cells (data not shown). When cells were cultured in serum-free medium, T53AScl2 and T53AScl4 cells expressed 2330 and 2110 copies, respectively, and T53 cells expressed 5256 copies (2.5-fold more) of *bcl*-2 transcripts per 10^5 cells at 48 h (Fig. 4B). At 48 h of cell culture, T53 cells produced an amount of *bcl*-2 transcripts comparable to T53AScl2 and T53AScl4 cells, when extracellular Tat was blocked by addition of a Tat antibody or PNU153429 to the culture medium of T53 cells (Fig. 4B), indicating that the greater *bcl*-2 expression of untreated T53 cells at 48 h was entirely due to the effect of extracellular Tat. The reduction of *bcl*-2 transcripts is specifically due to the pres-

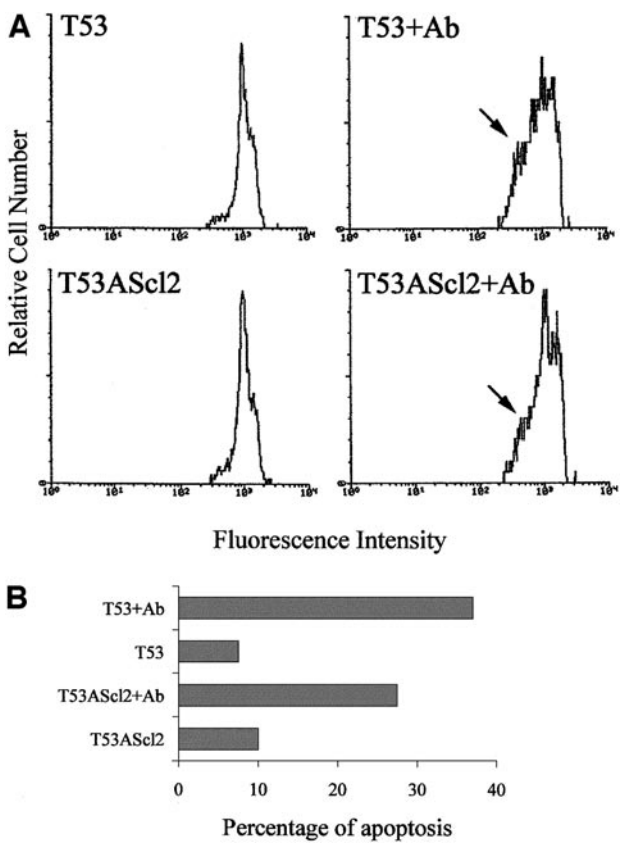

FIG. 3. Evaluation of apoptotic death by flow cytometry in T53 and T53AScl2 cells. Cells were cultured in serum-free medium and harvested at 48 h after the addition of a rabbit anti-Tat polyclonal antibody (Ab). (A) T53 and T53AScl2 cells were untreated or treated with the anti-Tat antibody, respectively. Percentage of apoptotic cells is shown in B. Significance of the results was calculated by χ^2 analysis. Values were T53 + Ab versus T53: $P < 0.00001$; T53AScl2 + Ab versus T53AScl2: $P < 0.00001$.

TABLE 2

Tumorigenicity of T53, T53AScl2, T53AScl4 Cells and Angiogenesis in Tumors

Cell lines	Tumorigenicity				Angiogenesis		
					Number of capillaries[c]		
	Mice with tumors/ inoculated mice	Tumor volume (mm^3)[a]	Mice with metastases/ mice with tumors	Total number of metastases[a,b]	Day 13	Day 24	Day 35
T53	6/6	1313 ± 344.2	4/6	33	193.8 ± 42	117.7 ± 21.2	111.6 ± 27.4
T53AScl2	4/4	210 ± 76.4*	2/4	2***	43.5 ± 9.2*	64.3 ± 13.7*	27.2 ± 8*
T53AScl4	4/4	445 ± 199.3**	2/4	11†	27.5 ± 9.2*	54.7 ± 23.6*	ND[d]

Note. Mice were inoculated subcutaneously with 5×10^5 cells per animal. Tumors appeared with a latency period of 2 to 6 days. Data show means ± SD and were statistically evaluated by Student's *t* test (*$P < 0.001$, **$P < 0.01$).

[a] Tumor volume and metastases were evaluated in mice sacrificed 30 days after cell inoculation.

[b] Metastases were located in lymph nodes, lungs, renal glomeruli, and heart. Data were statistically evaluated by χ^2 analysis (***$P < 0.0005$, †$P < 0.05$).

[c] In a separate experiment, the number of capillaries was counted in 12 different fields of each tumor at 13, 24, and 35 days after cell inoculation.

[d] ND, not done.

ence of the AS *tat* gene in T53AScl2 and T53AScl4 cells, as shown by the results obtained with T206 cells which contain the *tat* gene, but do not express Tat protein. In fact, both T206 cells and stable clones of T206AS cells, cultured in serum free-medium, produced from 400 to 500 copies of *bcl*-2 transcripts per 10^5 cells (Fig. 4B), suggesting that the expression of an AS *tat* gene does not induce nonspecific alterations in cellular physiology. The decrease of *bcl*-2 expression in all cell cultures at 72 h (Fig. 4B) is likely due to T53, T53AScl2, and T53AScl4 cells reaching confluence and ceasing proliferation, while in T53 cells treated with Tat antibody or PNU153429 the low value of *bcl*-2 transcripts is probably dependent on the progression of the apoptotic process (Figs. 2E and 2F). In the presence of a similar amount of *bcl*-2 RNA, T53AScl2 cells survive (Fig. 2A), whereas T53 cells, under conditions of neutralization of extracellular Tat by Tat antibody or PNU153429, develop apoptosis (Figs. 2E and 2F), suggesting that extracellular Tat released by T53AScl2 cells protects them from apoptosis by a metabolic pathway independent of *bcl*-2. This evidence indicates that cell survival and proliferation are supported by extracellular Tat through multiple mechanisms.

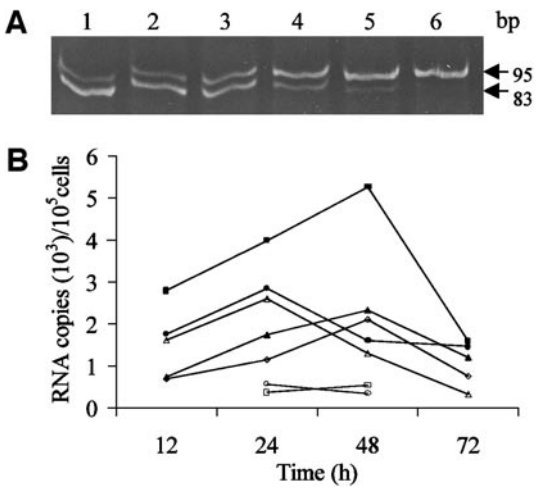

FIG. 4. Quantification of *bcl*-2 gene expression by qRT-PCR. (A) Cytoplasmic RNA, extracted from 10^5 T53AScl2 cells, after 24 h of culture in serum-free medium, was subjected to reverse transcription and amplified together with a known number of molecules of competitor RNA (10,000, 5000, 2500, 1250, 625, and 312, from lane 1 to 6). The wt (95 bp) and deleted competitor (83 bp) PCR products are indicated by arrows. In this experiment, 1680 wt *bcl*-2 mRNA copies were calculated (*20*) to be present in T53AScl2 cells. (B) Production of *bcl*-2 mRNA by cells after 12, 24, 48, and 72 h of culture in serum-free medium. Cytoplasmic RNA was extracted from 10^5 cells. Untreated T53 cells (■); T53 cells treated with an anti-Tat antibody (△) or PNU153429 (●); T53AScl2 (▲) and T53AScl4 (◇) cells; T206 (□) and T206AScl2 (○) cells.

Thus, inhibition of Tat in T53AS cell clones results in a reduced expression of *bcl*-2, which may be responsible for the limited tumor volume, metastasis formation, and angiogenesis observed in mice injected with the T53AS cells. These data confirm those of Nor *et al.* (*23, 24*) indicating that *bcl*-2 is involved in angiogenesis and suggest that Tat-induced *bcl*-2 expression may significantly contribute to the angiogenic effect of Tat. Moreover, relative to the importance of extracellular Tat in oncogenesis and angiogenesis, these results confirm and extend a previous investigation showing that mice inoculated with T53 cells and treated with PNU153429 developed smaller and less vascularized tumors and a smaller number of metastases than untreated control mice (*25*). The present study supports a contribution of Tat, through *bcl*-2 activation, to angiogenesis and oncogenesis during HIV-1 natural infection. Inhibition of Tat activity with immunoprophylactic, pharmacological, and gene therapy approaches may therefore help to prevent and control tumor development in the course of AIDS.

Materials and Methods: Cell cultures, DNA transfection, RNA extraction, and DAPI staining of cultured cells. T53 and T206 cell lines were established from adenocarcinomas of skin adnexa of *tat* transgenic mice, as

previously described (*10, 11*). T53AS and T206AS clones were obtained after transfection of cells with the plasmid pRPneo-*tat*/AS (2.5 μg/10^6 cells), containing HIV-1 *tat* cDNA in antisense orientation under control of the SV40 promoter (*6*). T53AS and T206AS clones were selected with 200 μg/ml of geneticin (G418; Sigma Chemical Co., St. Louis, MO). Cells were cultured in DMEM-F12 medium (Gibco BRL, Grand Island, NY) with 10% FBS (Gibco BRL). DNA transfection was performed on subconfluent T53 and T206 cells by the calcium phosphate precipitation technique (*9, 16*). Growth characteristics and plasminogen activator activity were carried out as previously described (*10, 11*). T53 cells (1×10^6) were treated in serum-free medium with rabbit polyclonal anti-Tat IgG (5 μg/ml; American Biotechnologies, Cambridge, MA) or with PNU153429 (5 μM; Pharmacia & Upjohn, Milan, Italy), a new polysulfonated distamycin A derivative (*12*). Cells were harvested at 12, 24, 48, and 72 h after addition of antibodies or PNU153429 and pellets were used for RNA extraction. Cytoplasmic RNA was extracted using an RNase mini kit (Qiagen, Santa Clarita, CA) according to the manufacturer's protocol. DAPI (4′,6-diamidino-2-phenylindole; Sigma) staining of cultured cells was carried out as described by Collins *et al.* (*8*). pRPc cells are mouse kidney cells transformed by BK virus early region DNA and were used as a negative control in the evaluation of intracellular Tat protein by flow cytometry.

Detection of Tat protein by flow cytometry. Exponentially growing cells (2×10^6) were harvested and permeabilized with 70% ethanol for 1 h at 4°C. Cytometric detection of Tat molecules was performed by immunofluorescence using a 1:10 dilution of a mouse anti-Tat monoclonal antibody (MAb; American Biotechnologies), followed by a goat anti-mouse IgG covalently linked to fluorescein (GAM-F1; Becton Dickinson, San Jose, CA). Staining was performed in 200 μl phosphate-buffered saline containing 1% bovine serum albumin (Sigma), at 4°C for 30 min. Nonspecific fluorescence was assessed with a nonspecific isotype-matched primary mouse MAb to cytomegalovirus (DuPont, Boston, MA) followed by GAM-F1 and with GAM-F1 alone. To quantify cell-associated fluorescence, before the assay the FACScan was calibrated by quantitative fluorescein microbeads (Flow Cytometry Standard Corp.). A standard curve was constructed by plotting the mean fluorescence intensity against the logarithm of the number of fluorescein molecules per bead. Samples were run in duplicate. Relative anti-Tat MAb binding sites per cell were obtained by dividing the number of fluorescein molecules bound per cell by the fluorescein-to-protein ratio of the anti-Tat MAb. All experiments were performed under conditions of saturation.

Evaluation of apoptosis by flow cytometry. Cells, maintained in culture in serum-free medium, were harvested at 48 h after addition of rabbit anti-Tat polyclonal antibody or PNU153429 and fixed in cold 70% ethanol at 4°C for 1 h. Flow cytometry studies were performed as described previously (*22*) using 10^4 cells per sample. Results were evaluated by χ^2 analysis.

Oncogenicity in nude mice and evaluation of angiogenesis. Four- to 8-week-old nude mice (Balb/c-nu/nu) were inoculated subcutaneously with 5×10^5 cells per animal and examined daily for the appearance of tumors. To evaluate tumor volume and metastases, mice were sacrificed 30 days after cell inoculation. To estimate the entity and extent of the vascular network in tumors, in a separate experiment animals were injected ip, at 13, 24 and 35 days after cell inoculation, with the fluorochrome Hoechst 33342 and killed after 1 min. Tumors were removed, frozen in liquid nitrogen, and sectioned at three different levels, and four fields of each level were observed under a UV microscope to count fluorescent vessels. The Hoechst 33342 vascular staining technique was used according to Smith *et al.* (*28*).

Primers used for mutagenesis and amplification. Three primers were used for the competitive analysis of mouse *bcl-2* mRNA expression: Bcl3, Bcl4, and Bcl4mod. Bcl3 (24-mer) (5′-CACACCTGGATCCAGGATAACGGA-3′, forward) maps at the 5′ end of exon 1 of the *bcl-2* gene; Bcl4 (26-mer) (5′-AGAGACAGCCAGGAGAAATCAAACAG-3′, reverse) maps at the 3′ end of exon 2; and Bcl4mod (44-mer) (5′-AGAGACAGCCAGGAGAAATCAAACAGAGGGCCATATAGTTCCAC-3′) maps at the 3′ end of exon 2 and was used for mutagenesis. This primer, during the PCR, generates a 12-bp loop in the single-strand template resulting in a deletion of the amplified fragment.

Synthesis of the bcl-2 competitor RNA. PCR-mediated mutagenesis was used to produce the pBcl-2α plasmid, containing a *bcl-2* deleted copy of the Bcl3–Bcl4 cDNA fragment. The 95-bp product, obtained after RT-PCR of total RNA from T53 cells, using the primers Bcl3–Bcl4, was amplified with the primers Bcl3 and Bcl4mod in order to obtain a fragment with an internal 12-bp deletion. PCR was performed in a 480 Thermal Cycler (Perkin–Elmer Cetus, Foster City, CA) under the following conditions of amplification: 94°C for 15 s, 35°C for 30 s, 72°C for 1 min for the first 6 cycles; 94°C for 30 s, 60°C for 30 s, 72°C for 1 min for the following 44 cycles. Final extension was at 72°C for 15 min. The amplified 83-bp product, which represents the competitor DNA, was treated for 1 h at 37°C with 2.5 U of T4 DNA polymerase, in order to generate a blunt-end fragment, and cloned into the *Srf*I site of the phagemid pCR-Script SK(+) (Stratagene, La Jolla, CA), to obtain plasmid pBcl-2α. *In vitro* mRNA was transcribed from the *Eco*RI-linearized pBcl-2α plasmid (1 μg) with T3 RNA polymerase (Riboprobe System-T3, Promega, Madison, CA) as recommended by the manufacturer. Synthesized RNA was resuspended in diethyl pyrocarbonate-treated water and

aliquots were immediately frozen in dry ice and stored at −80°C. Competitor RNA copy number was exactly quantified by spectrophotometric analysis, gel electrophoresis, end-point dilution RT-PCR, and Poisson distribution analysis of the last dilution giving a positive score. The sensitivity of RT-PCR was equivalent to retrotranscription of two copies of competitor RNA, as evaluated by spectrophotometric analysis.

Quantitative RT-PCR. For qRT-PCR, a known number of competitor RNA copies (diluted twofold from 10,000 to 312) and a constant amount of wild-type *bcl*-2 RNA (corresponding to 10^5 cells) were simultaneously subjected to reverse transcription, using the cDNA Cycle kit (Invitrogen Corp., San Diego, CA) in the presence of 20 pmol of Bcl4 antisense primer (final volume 20 μl). The resulting cDNA was amplified with 30 pmol of Bcl4 and 50 pmol of Bcl3 primers in a 9600 Thermal Cycler (Perkin–Elmer) using the following conditions of amplification: 50 cycles at 95°C for 15 s, 60°C for 15 s, 72°C for 10 s (with an increase of 1 s per cycle in the third step) and a final incubation at 72°C for 15 min. The amplified products were analyzed on 10% polyacrylamide minigels at 180 V for 45 min for optimal separation of the 95-bp *bcl*-2 wt fragment from the 83-bp *bcl*-2 deleted fragment. After ethidium bromide staining, fluorescent bands were scanned using a video densitometer (Bio-Rad Laboratories, Hercules, CA) and the RNA copy number was calculated as described by Menzo *et al.* (*20*).

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