Journal of Cellular Biochemistry 101:1148–1164 (2007)

Alternative Production of Bcl-2 and Bax by Tumor Cells Determines the Rates of In Vivo Tumor Progression: Suggested Mechanisms

Galina I. Deichman,* Natalya A. Dyakova, Valentina A. Matveeva, and Lydia M. Kashkina

Laboratory of Antitumor Immunity, Institute of Carcinogenesis, N. N. Blokchin Cancer Research Center, Russian Academy of Medical Sciences, 115478 Moscow, Russia

Abstract The hypothesis tested in the study suggests that mechanisms of the earlier described delayed or accelerated tumor progression may be regulated by the antiapoptotic and proapoptotic cellular programs activated in stress reactions of transformed cells to the host normal cellular environment. Therefore, spontaneously transformed hamster cell line STHE, its bcl-2-transduced line STHE-Bcl-2, and 64 of their descendant tumor cell variants naturally selected in two in vivo regimes (local tumor growth versus dissemination) were examined. The role of Bcl-2 and the possible activation of endogenous death-signaling Bax, Ras, and HSP90/HSP70 stress proteins in STHE (Bcl-2+/−) tumor cell variants were studied in dynamics of in vivo tumor progression. The data demonstrate: (1) Immediate in vivo activation of Bax and of HSP90/HSP70 stress proteins in disseminated STHE cells on the background of accelerated tumor progression; (2) No immediate activation of Bax and the gradual downregulation of Bcl-2 in STHE-Bcl-2 cells on the background of delayed tumor progression; (3) Alternative and mutually suppressive character of Bcl-2 and Bax expression in both regimes of tumor progression; (4) In the later stages of tumor progression, the regular transit of the initial Bcl-2 antiapoptotic, Bax-suppressing program, and the delayed tumor progression towards Bcl-2 loss, activation of Bax, and acceleration of tumor progression. Thus, the delay of tumor progression is apparently determined by the ability of Bcl-2-expressing tumor cells to extinguish the cell-damaging environmental stress signals and Bax activation, while its acceleration correlates with Bcl-2 loss, activation of proapoptotic Bax, and tumor cells damage. J. Cell. Biochem. 101: 1148–1164, 2007.

Key words: Bcl-2; Bax; Ras; HSP90/HSP70; tumor progression: acceleration and delay; HP-phenotype

Genetic and epigenetic changes of transformed cells during in vivo multistep tumor progression towards higher malignancy occur under conditions of natural selection of tumor cell variants more resistant to almost any type of cell DNA damaging factors, in particular to the antitumor stress reactions of the host normal cellular environment. These changes are apparently highly influenced by the host vital, organ-specific, hormonal, differentiation, and death signals and involve a set of insufficiently studied biochemical reactions of tumor cells (TC) to contact interaction with the host cellular environment [Nowell, 1986; Dotto et al., 1988; Weinberg, 1989; Bauer, 1996; Witz et al., 1996; Hanahan and Weinberg, 2000; Klein, 2002; Roskelley and Bissell, 2002; Fidler, 2003; Klein, 2004]. Surprisingly, the very beginning (first hours and days) of in vivo experience of syngeneic TC is followed by mass death and damage to these cells, especially high (near 100%) under conditions of dissemination [Fidler, 1970; Poste and Fidler, 1980; Price et al., 1984, 1986].

The majority of the host antitumor reactions in vivo apparently represent the ancient, immunologically nonspecific rejection reactions directed against the cells recognized as diverse (mutant, senile, damaged, etc.) [Klein, 2004;

Grant sponsor: Russian Foundation for Basic Research; Grant numbers: 99-04-48358a, 02-04-48232a, 05-04-49439a.

*Correspondence to: Prof. Galina I. Deichman, Laboratory of Antitumor Immunity, Institute of Carcinogenesis, N. N. Blokchin Cancer Research Center, Russian Academy of Medical Sciences, Kashirskoye shosse 24, 115478 Moscow, Russia. E-mail: antitum@space.ru

Received 14 December 2005; Accepted 21 November 2006

DOI 10.1002/jcb.21242

Klein and Klein, 2005]. Transformed cells (TrC), as compared with normal cells, appeared to be even more sensitive to the intercellular and intracellular death signals, especially those mediated by reactive oxygen and nitrogen species [Bauer, 1996; Hanusch et al., 2000; Heigold et al., 2002]. As shown earlier, during in vivo natural selection, TrC regularly acquire increased H_2O_2-catabolizing activity and the resistance to damage by hydrogen peroxide [Deichman and Vendrov, 1986; Deichman et al., 1989] and to hypoxia [Graeber et al., 1996] together with significantly higher tumorigenicity.

During the last about 15 years, we have addressed our studies to identification of the earliest regular phenotypic and biochemical changes acquired by Syrian hamster in vitro TrC of different origin (spontaneous and virus-induced transformation) and particularly, during their in vivo subcutaneous (sc) growth and intravenous (iv) dissemination. The dynamics of in vivo natural selection of the descendant TC variants towards higher tumorigenicity and metastasizing was studied [reviewed by Deichman, 1988, 2000, 2002]. It has been demonstrated that besides 10–100-times increased tumorigenic activity (usually not yet followed by spontaneous metastasizing activity), the acquired resistance of TC to cytotoxic activities of macrophages (Mph) and NK cells correlated with their: (1) increased H_2O_2-catabolizing activity ($H_2O_2{}^{CA}$); and (2) acquired ability to release E_2 prostaglandins (PGE^S) in contact interaction with NK cells [Deichman et al., 1989a; review by Deichman, 1988]. Unexpectedly, the co-expression of these two biochemically different cell properties was regularly acquired by the descendant variants of TrC in vivo at almost the same time of sc tumor growth during 100 ± 20 days. We designated these stable changes as ($\underline{H}_2O_2{}^{CA} + \underline{P}GE^S$) phenotype (abbreviation HP-phenotype) and considered them as a marker of possibly the earliest (premetastatic) stage of in vivo tumor progression [review by Deichman, 2002].

Unexpectedly, with the use of HP-phenotype as a stage marker iv disseminated STHE cells demonstrated high acceleration of tumor progression as compared with sc grown STHE cells. In contrast, striking delay of tumor progression was demonstrated in the case of bcl-2-transduced STHE cells, disseminated iv or grown sc [Deichman et al., 1998; Dyakova et al., 2001; rev. Deichman, 2002]. However, the mechanisms responsible for the delay in tumor progression of Bcl-2-expressing TrC, as well as for acceleration of this process in the case of Bcl-2-non-protected STHE cells remained unclear. Therefore, our present studies were addressed to the mechanisms of the in vivo delayed and accelerated tumor progression and particularly to the suggested role of the in vivo activation of antiapoptotic and proapoptotic programs of Bcl-2-protected, or not protected TrC in their direct contact interaction with the host normal cellular environment.

Published data on the role of antiapoptotic and proapoptotic tumor cell activities in tumor progression are controversial. Several clinical observations connect Bcl-2 expression with favorable prognosis and suggest delayed cancer progression. The loss of Bcl-2 in human breast cancer increases TC death and proliferation [van Slooten et al., 1998] and allows accumulation of genetic alterations [Sierra et al., 2000]. The antiapoptotic Bcl-2 and proapoptotic Bax activities have been considered as independent cell functions [Knudson and Korsmeyer, 1997]. Hepatic carcinogenesis related to either c-myc, or to TGFα were both delayed and altered by Bcl-2 [de La Coste et al., 1999; Vail et al., 2001]. Also, Bcl-2 inhibited the development of F-MLV-erythroleukemias [Howard et al., 2001] and lymphoma development in Bax transgenic mice [Luke et al., 2003]. In the majority of experimental studies, the pro-survival activity of Bcl-2 or mutational inactivation of Bax are usually considered as factors a priori promoting in vivo survival of TC and thus, tumor growth and progression [Haldar et al., 1994; Knudson and Korsmeyer, 1997; Ionov et al., 2000; rev. in Adams and Cory, 1998; Zornig et al., 2001; Yin, 2006; van Delft and Huang, 2006]. On the other hand, in p53-deficient mice acceleration of tumor progression by Bax has been shown [Knudson et al., 2001].

The findings presented in this study support the hypothesis that dynamics of in vivo tumor progression (accelerated or delayed) correlate with the ability of TC to follow either their Bcl-2-antiapoptotic or Bax-proapoptotic cellular programs; immediate in vivo activation (or suppression) of a set of endogenous death-signaling proteins, such as Bax, Ras, and HSP90/HSP70 stress proteins is shown. In contrast to a widely accepted opinion, activation of endogenous Bax and correspondingly, worse

survival of iv disseminated TC in vivo correlated with highly accelerated tumor progression, while expression of the prosurvival Bcl-2 activity inhibited activation of Bax and significantly delayed tumor progression. In vivo expression of Bcl-2 and Bax proteins appeared to be alternative and mutually suppressive in both STHE (Bcl-2+/−) cell lineages and in both regimes of tumor progression.

MATERIALS AND METHODS

Cells

Syrian hamster embryo cells spontaneously transformed in vitro (STHE cell line), its bcl-2-transduced cell line (STHE-Bcl-2), and a collection of 64 of their variant descendant cell lines, naturally selected in two in vivo regimes (local sc growth versus dissemination) and then transferred in tissue culture were studied. Details of STHE cells transduction with retroviral vector carrying cDNA for human Bcl-2 gene (pLBSN), kindly performed by M. Nikiforov and A. Gudkov, as well as some original characteristics of these cell lines were presented elsewhere [Deichman et al., 1998]. Briefly, both in vitro maintained STHE parental cell lines, (Bcl-2+/−) were non-immunogenic, low-clonogenic, low-tumorigenic, non-metastasizing spontaneously, and characterized by low basic levels of endogenous Bax, Ras, and HSP90 and HSP70 stress proteins production. After transduction with bcl-2, high and stable level of Bcl-2 protein production was characteristic for in vitro growing STHE-Bcl-2 cell line. Indirect data (high susceptibility to oxygen stress, to γ-irradiation, and to cytotoxicity of Mph and NK cells) suggested that STHE parental cell line (examined before any in vivo experience) retain wt p53 activity. Cell lines were cultured in vitro as monolayers with the use of DMEM culture media, adding 7–10% fetal calf serum and antibiotics.

In Vivo Natural Selection of STHE (Bcl-2+/−) Variant Tumor Cells Lines From the sc Growing or iv Disseminated TC

In contrast to conditions of sc local tumor growth, where descendant TC variants could be isolated only after the appearance of palpable tumor nodules, the high experimental advantage of the use of iv dissemination of TrC is the possibility to isolate the surviving descendant TC variants from different host tissues (for instance, from lung tissues) at any time after iv inoculation of TrC, that is, long before the appearance of visible tumor nodules in these tissues [Deichman et al., 1994; Dyakova et al., 2001]. Descendant variants of STHE and STHE-Bcl-2 cells were isolated at different time intervals after either sc or iv inoculation of normal adult Syrian hamsters in accordance with Figure 1. While sc-transplanted TrC undergo relatively moderate environmental stress, when about 10–15% of inoculated TC survive and grow, the mass death (near 100%) of the same cells take place under conditions of dissemination. It apparently means that environmental stress and, correspondingly, the host in vivo selection pressure on iv

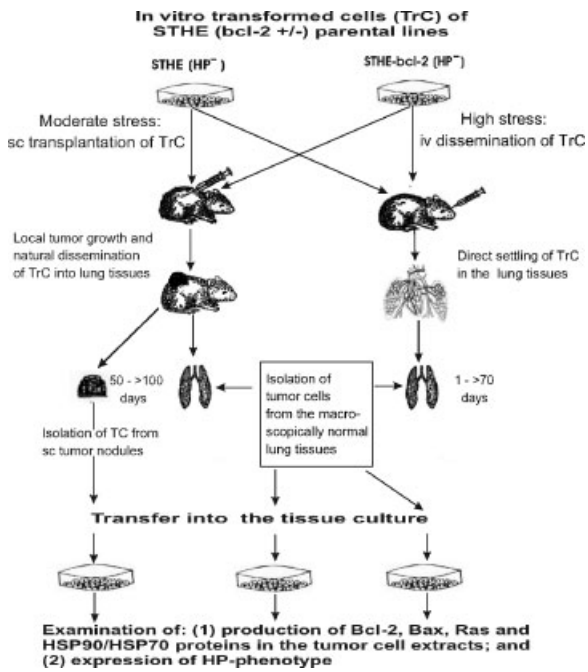

Fig. 1. Modeling of in vivo natural selection of Syrian hamster in vitro transformed cells under conditions of moderate or high environmental stress (local tumor growth vs. dissemination). Single cell suspensions of two in vitro transformed tissue culture cell lines of one origin (STHE and STHE-Bcl-2), in vitro spontaneously transformed and Bcl-2 transduced, never before selected in vivo, low-malignant, not expressing HP-phenotype, were inoculated subcutaneously (sc) (2.0×10^4/0.2 ml), or intravenously (iv) ($2.0–5.0 \times 10^6$/1.0 ml) into normal adult Syrian hamsters. At different times of near 50 and 100 or more days of sc growth and at 1 to >70 days after iv inoculation, 3–5 animals were sacrificed and tumor cells (TC) were isolated from individual sc tumor nodules; iv disseminated TC were isolated from macroscopically normal lung tissues of individual inoculated animals. Isolated descendant TC variant were grown in tissue culture, extracted, and examined in sandwich ELISA for the production of Bcl-2, Bax, Ras, and HSP90/HSP70 and on the expression of HP-phenotype (details in Materials and Methods).

disseminated TrC is significantly higher than in the case of sc growing TC [discussed by Deichman, 2002].

Isolation of TC Variants From sc Grown Tumor. Briefly, the procedure used for natural selection of STHE and STHE-Bcl-2 descendant TC variants from the sc growing tumors in normal Syrian hamsters was standard (Fig. 1, left side) including: (a) the number of TrC in the single cell suspensions (2.0×10^4/0.2 ml/point) sc inoculated into 5–6 normal 2-month old hamsters; and (b) 45–60 days of in vivo duration of each sc growth cycle. All animals in these and subsequent experiments were maintained and used in accordance to the State Ethical Guidelines for Animal Care. At 45–60 days of sc tumor growth, the animals were sacrificed using of ethyl ether. Tumor cells of 3–6 or more sc tumor nodules, each from individual animal of the same group, selected randomly, were excised in sterile conditions, washed free from blood, cut mechanically, and treated with Versene solution (2% EDTA solution, Sigma); cell suspensions were washed in PBS, resuspended in culture medium, and grown as monolayers. After 3–5 in vitro passages, the isolated TC variants were, as a rule, completely cleared from the admixture of the host stromal cells and could be further examined as a descendant variants of the parental cell line. The total duration of in vivo experience of each tumor cell line passaged sc in several in vivo cycles of sc growth was summarized.

Isolation of Descendant TC Variants From Lung Tissues of Hamsters iv Inoculated With TrC. For this purpose, the single cell suspensions of TrC prepared from both in vitro grown tissue cultures of STHE (Bcl-2+/−), were inoculated iv ($2.0–5.0 \times 10^6$/1.0 ml/hamster) into the retroorbital venous cavity of 2-month-old normal Syrian hamsters (Fig. 1, right side). At different time intervals after iv inoculation (in the present study from 1 to 70 days), 3–6 animals from the group were sacrificed under ethyl ether treatment and the individual, macroscopically normal lungs (free from visible metastasis) were excised in sterile conditions, washed free from the blood, cut mechanically, treated with 2% EDTA solution, centrifuged, and resuspended in DMEM culture medium supplemented with 10% fetal calf serum and antibiotics. The majority of TC lines isolated from the lung tissues of iv inoculated animals were obtained in two following steps: (1) enrichment in the lung cell suspension of disseminated TC using a Percoll gradient (20–24%) and (2) growth in tissue culture. During the subsequent few culture media changes and in vitro passages, the admixture of normal stromal and lung cells are gradually eliminated, while the lung-isolated TC dominantly grew in tissue culture as a monolayer. All variants of TC lines isolated from sc tumors and from the lung tissues are stored under liquid nitrogen.

Expression of ($\underline{H}_2O_2^{CA} + \underline{P}GE^S$) (HP) phenotype

Acquisition of HP-phenotype was determined for the parental and for each in vivo naturally selected descendant TC variant according to Deichman et al. [1998]. This included the simultaneous in vitro examination of the following two TC properties: (1) the antioxidant H_2O_2-catabolizing ($H_2O_2^{CA}$) activity; and (2) the ability to release PGE_2 (PGE_2^S) in culture medium immediately in response to contact interaction with NK cells. Briefly, $H_2O_2^{CA}$ was determined using the luminol-dependent chemiluminescence (LDC) test in standard tumor cell extracts (adjusted to 2.0 mg/1.0 ml protein), treated with standard doses of H_2O_2. The thermostatted Biolumate model 9500 luminometer, Berthold, Gossheim, Germany) was used. PGE_2-releasing activity (PGE^S) of in vivo selected TC variants was determined by either RIA [Young and Knies, 1984] or by biological test [Kluchareva et al., 1992]. In both tests, as PGE^S controls, intact TC and TC pretreated with indomethacin (Serva) (20γ/1.0 ml, 2 h at 37°C) were examined in parallel.

Modified Sandwich ELISA Used for Determination of Bcl-2, Bax, Ras, HSP90, and HSP70 Proteins in TC Extracts

Monoclonal antibodies (MABs). The following MABs of mouse IgG_1 isotype (all obtained from BD Bioscience, Pharmingen) were used: anti-Bcl-2 (cat. No. 610539; clone 7); anti-HSP90 (cat. No. 610419, clone 68); anti-HSP70 (cat. No. 610608; clone 7); purified mouse anti-Bax (cat. No. 556467, clone 6A7); mouse anti-Ras (cat. No. 610002, clone 18); horseradish peroxidase (HRP)-conjugated rat anti-mouse IgG_1 MAB (cat. No. 559626, clone X56). In accordance with the technical data of BD Bioscience, Pharmingen, the specificity of MABs against HSP90 (clone 68) and HSP70 (clone 7) was confirmed using Western blots (WB) with lysates of HeLa cells demonstrating

that the corresponding antibodies reveal antigens of 90 kDa and 70 kDa, correspondingly, and do not show cross reactions. Specificity of anti-Bax MABs (clone 6A7) was challenged in WB using Daudi human B cells and mouse thymocytes, which both revealed Bax as a 21-kDa band. Specificity of anti-Bcl-2 MABs were examined in a WB test using Jurkat cell lysate, which revealed Bcl-2 as a 26-kDa band. Specificity of anti-Ras MABs (clone 18) was confirmed in WB using A431 cell lysate, which reveal Ras as a 21-kDa band. The titers of anti-HSP90 compared with anti-HSP70 MABs in ELISA tests examined in the present study in extracts of the same cell lines, as well as in their variant cell lines, appeared to be practically the same. Therefore, in Figures, they were presented as the titer of anti-HSP90/HSP70 MABs.

Preparation of Cellular Extracts for ELISA and WB Tests

Monolayer tissue cultures of in vitro grown TrC of the parental STHE and STHE-Bcl-2 cell lines and of their in vivo naturally selected TC variants were taken from the glass using Versene solution (EDTA, Sigma), twice washed with PBS and extracted by freezing and thawing at −20°C in PBS followed by centrifugation (8,000*g*, 15 min, 4°C) and the addition of Triton X-100. For WB, the tumor cells extracts prepared by freezing and thawing as for ELISA were in addition treated with 1% SDS and heating (95°C), followed by electrophoresis in accordance to Laemmli [1970] and preparation of Western blots. Total protein content in TC extracts was determined under cold conditions according to Bradford, adjusted to 2.0 mg/1.0 ml, distributed in Eppendorf vials in a volume of 50 μl/sample and frozen at −20°C until use. Before use in reactions, extracts were diluted 1:10 (to 0.2 mg/1.0 ml).

WB assay was mainly used for identification of the proteins under study by molecular weight. However, due to the unforeseen loss of the proteins during the preparation of WB, and particularly during their additional treatment with SDS and heating, and then in electrophoresis and transfer, determination of proteins in WB is rather more qualitative than quantitative. Besides, the treatment of cell extracts with SDS breaks the dimerized and heterodimerized forms of Bcl-2 and Bax and thus apparently alters the real composition of these protein complexes in the tumor cell extracts. In contrast to WB, in ELISA, one deals with significantly less damaged proteins and identifies them mainly in their free forms [especially as concerns MABs used in the present study, Hsu and Youle, 1997]. These technical differences make determination of proteins in ELISA, as compared with WB, more sensitive and quantitative, especially in cases, when the expression and production of these proteins varied widely. Therefore, in the present study for evaluation of the level of proteins expressed in the compared cellular extracts, we used mainly ELISA in its sandwich modification and the titration of the corresponding MABs against one standard dose of cell extract; in some experiments, the titration of standard cell extracts (diluted 1:2–1:8) against one dose of MABs were used.

ELISA (Enzyme-Linked Immunosorbent Assay)

Slightly modified sandwich ELISA was used for determination of Bcl-2, Bax, Ras, and HSP90/HSP70 stress proteins in cellular extracts of TC lines. In accordance to a modification of sandwich ELISA [Valdes et al., 2003], the same MABs were used as a capture (bottom) component, immobilized onto the wells of 96-well tissue culture plates (Costar) and as upper detection antibodies. The use in ELISA of the same MAB as a capture and as upper antibody appears useful in the cases when the cell proteins under study normally exist in the free, as well as in the aggregated forms of homo- and heterodimers; the latter is especially true for Bcl-2 and Bax [Oltvai et al., 1993; Yin et al., 1994; Hsu and Youle, 1997; Hirotani et al., 1999; Yin, 2006; van Delft and Huang, 2006].

In majority of sandwich ELISA tests used in the study, the capture antibody was diluted in carbonate buffer (pH 8.4) 1:4,000; the upper detection antibody of the same specificity was twofold diluted in PBS from 1:1,000–1:2,000 to 1:16,000–1:32,000. After blocking with 1% BSA in PBS and washings, the wells were covered with 0.1 ml of cellular extract (adjusted to total protein 0.2 mg/1.0 ml) or with 0.1 ml of its diluted (1:2–1:8) samples. Then, after overnight incubation at 4°C and washings, the wells were coated with different dilutions of upper (detection) MABs of the same specificity using three wells for each dilution of antibodies. As controls, the same dilutions of MABs free from the cell extracts, as well as cell extracts free from antibodies, were used. As other control (if necessary), coating the wells with normal

serum, instead of the specific MABs was included. The quantification seems to be linear in the interval between 1:2,000 to 1:8,000 dilutions of anti-Bcl-2 and anti-Bax MABs. This makes it possible to semi-quantitatively compare the levels of particular proteins determined in different standard cell extracts by either of two approaches, that is, by the titration of MABs against one dose of TC extracts, or by the titration of TC extracts diluted 1:2–1:8 against one or more doses of MABs. The modified sandwich ELISA used in the study for determination of Bcl-2, Bax, Ras, and HSP90/HSP70 mainly by the titration of upper MABs against different undiluted TC extracts, repeated 2–3 times, demonstrated stable and highly reproducible results.

The rat antimouse HRP-labeled antibodies at dilution 1:4,000 and o-phenylenediamine dihydrochloride (Sigma) were used for the display of ELISA reaction (with absorbance at 492 nm) with a Uniplan reader (Pikon, Russia). The significant difference ($P < 0.001$) in the mean ($M \pm m$) optical densities (OD) (usually near or equal to 0.100 OD units) between the highest dilution of corresponding MABs against the standard dose of the cell extracts and the parallel mean OD values of control wells containing the same dilutions of MAB, free from the cell extract (minus OD of cell extract free of antibodies) was considered as an end-point titer of the upper MAB reaction in sandwich ELISA characterizing the relative quantity of the specific protein in the given cell extract. The use of anti-Bcl-2 and anti-Bax MABs in ELISA assay with the same TC cell extracts, undiluted or diluted 1:2–1:8 allow evaluation of Bcl-2/Bax and Bax/Bcl-2 indices of the compared cell lines and thus, their dynamic changes in the same cell lines during tumor progression/examples in Tables I and II.

RESULTS

The experimental approach exploited in our present studies is based on the use of: (1) spontaneously transformed non-immunogenic hamster cell line STHE, protected or unprotected by Bcl-2 against apoptotsis and their in vivo naturally selected TC variants; (2) two standard regimes of tumor progression, physiological for in vivo tumor development and natural selection of TC variants (local tumor growth vs. dissemination) (Fig. 1). These regimes are significantly differing in the level of the host antitumor environmental stress (moderate or high), the corresponding better or worse TC survival and the dynamics of tumor progression; (3) mainly sandwich enzyme-linked immunosorbent assay (ELISA) for detection of the possible in vivo activation of such endogenous cell proteins as Bcl-2, Bax, Ras, and HSP90/HSP70; in limited number of experiments, Western blot (WB) assay was used; (4) HP-phenotype as a marker of the early (pre-metastatic) stage of in vivo tumor progression.

In the series of experiments, we compared the use of WB and of sandwich ELISA for determination of the significantly varied quantities of Bcl-2 and Bax proteins production by STHE parental (Bcl-2+/–) cell lines and their in vivo selected TC variants. As shown in WB (Fig. 2A–C), Bcl-2 and Bax proteins were determined as p26 and p21 bands, correspondingly, with the

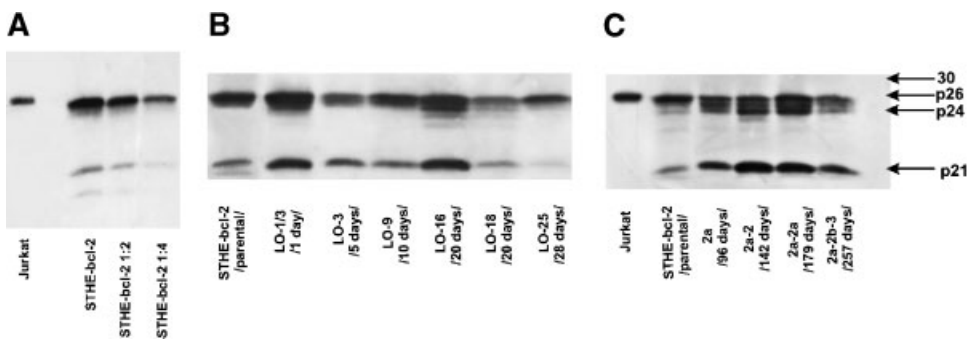

Fig. 2. Western blots of Bcl-2 and Bax expressed in STHE-Bcl-2 cell line and its in vivo selected tumor cell variants. **A**: Titration (1:2–1:4) of Bcl-2 in extract of the parental STHE-Bcl-2 cell line against anti-Bcl-2 MAB, clone 7 (1:500); (**B**) Expression of Bcl-2 p26 and of Bax p21 and p24 in STHE-Bcl-2 cells and their tumor cell variants lung-isolated at 1–28 days after iv dissemination of the parental cell line (anti-Bcl-2 MAB, clone 7, 1:500); (**C**) Expression of Bax p21 and p24 and of Bcl-2 p26 in extracts of STHE-Bcl-2 parental cell line and its variant tumor cells lines isolated from sc growing tumors at different times of 96–257 days (anti-Bax MAB 6A7, 1:500) (details in Materials and Methods).

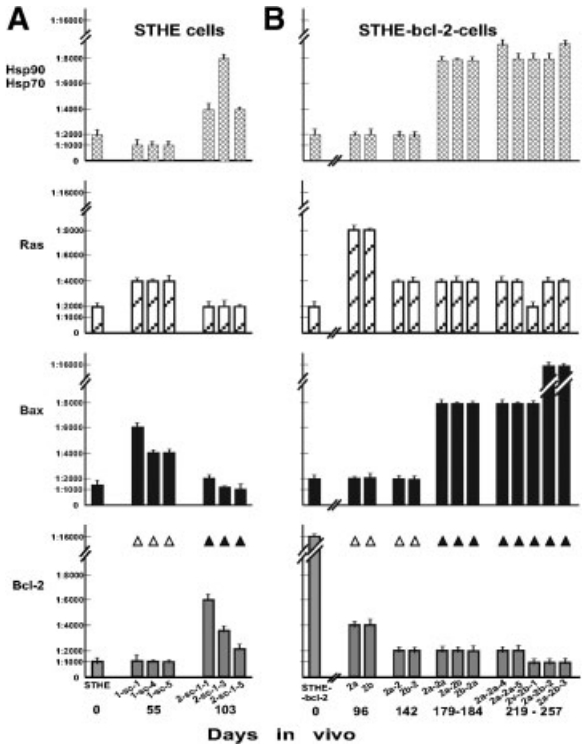

Fig. 3. **A**, **B**: Dynamics of Bcl-2, Bax, Ras, and HSP90/HSP70 production by STHE cells and STHE-Bcl-2 cells under conditions of sc tumor growth and acquisition of HP-phenotype (sandwich ELISA data). Single cell suspensions of in vitro spontaneously transformed tissue culture cells of STHE line (A) and STHE-Bcl-2 line (B) were inoculated sc (2.0×10^4/0.2 ml) into normal Syrian hamsters. At different times of in vivo sc growth, the descendant variant cell lines were isolated from the sc tumor nodules of 3–5 individual animals, grown in vitro in tissue culture, extracted, and examined. Production of Bcl-2, Bax, Ras, and HSP90/HSP70 by the parental and descendant variants of cell lines were tested in sandwich ELISA with the corresponding MABs diluted 1:1,000–1:2,000 to 1:16,000–1:32,000 (axis of ordinates) and on the expression of HP-phenotype. Abscissa—the denominations of the individual cell lines tested and the duration (in days) of their in vivo experience period; 0 point represents the levels of corresponding proteins in the parental TrC before inoculation in vivo (for details see Materials and Methods). Designations: ▒—Bcl-2; ■—Bax; ▩—HSP90/HSP70; ▨—Ras; ▲—HP^+ phenotype; △—HP^- phenotype.

use of anti-Bcl-2 MAB (clone 7) and anti-Bax MAB (6A7). In all WB tests, due to the known high homology and co-immunoprecipitation of these proteins by anti-Bcl-2 and anti-Bax MABs, the cross-reactivity between Bcl-2 and Bax proteins was regularly observed with either of these MABs. However, in WB, in contrast to ELISA, even the highest levels of the corresponding proteins present in the TC extracts could be determined using relatively high doses of MABs (diluted not more than 1:500), while lower doses of these proteins were less detectable (Fig. 2A). In contrast, in the modified sandwich ELISA, Bcl-2 and Bax proteins in the same TC extracts could be easily detected using significantly lower doses of MABs (diluted up to 1:16,000) (Fig. 3A,B). Thus, due to the higher sensitivity of ELISA, as compared with WB, the former apparently provides better possibilities to detect the lower quantities of these proteins in the TC extracts. Therefore in subsequent experiments, where we had to compare different quantities of Bcl-2, Bax, Ras, and HSP90/HSP70 proteins produced by the large number of TC variants at the different stages of tumor progression, we chose to use ELISA in its most sensitive sandwich modification (details in Materials and Methods).

In the first series of experiments (Fig. 3A,B), groups of 2-month-old normal Syrian hamsters were inoculated sc with the parental STHE and STHE-Bcl-2 cells and at different time intervals, the descendant TC variants were isolated from the sc developing tumor nodules and grown in vitro in tissue culture (Material and Methods). As shown in Figure 3A, on the 55th day of in vivo sc growth of STHE cells (the end of the first in vivo passage of TrC) on the background of the non-expressed HP-phenotype (empty triangles) and of the low basal levels of Bcl-2 production all three isolated STHE TC variants demonstrated the increase of Bax and Ras production. This picture changed during the second in vivo passage of these cells (ended at 103 days of sc growth), when acquired expression of HP-phenotype (black triangles) coincided with downregulation of Bax and Ras and the moderately increased levels of endogenous Bcl-2 and HSP90/HSP70 production.

In the case of STHE-Bcl-2 cells, the picture appeared to be quite different (Fig. 3B). During, 257 days of in vivo sc tumor growth (during five in vivo sc TC passages), the initially high level of Bcl-2 protein production gradually decreased, while production of endogenous Bax and of HSP90/HSP70 was simultaneously activated and greatly increased. These dynamic changes were registered in the sandwich ELISA by twofold dilutions of anti-Bcl-2 and anti-Bax MABs (diluted 1:2,000–1:32,000), challenged against the undiluted TC extracts of 13 different cell lines (Fig. 3B, two lower panels).

The picture was principally the same when some of the same cellular extracts of TC (diluted 1:2–1:8) were challenged against one dose of the same MABs (diluted 1:4,000) (Table I). In the

TABLE I. Gradual Loss of Bcl-2 by STHE-Bcl-2 Tumor Cell Variants and Simultaneous Activation of Endogenous Bax Production in Dynamics of In Vivo Tumor Progression

STHE-Bcl-2 cell variants[a]	Duration (in days) of in vivo tumor progression	Anti-Bcl-2 MAB (clone 7) Extract dilutions 0	1:2	1:4	1:8	Anti-Bax MAB (6A7) Extract dilutions 0	1:2	1:4	1:8
Parental	0	**0.904 ± 0.005**[b] **P < 0.001**	**0.804 ± 0.003** **P < 0.001**	**0.602 ± 0.010** **P < 0.001**	0.380 ± 0.005 NS	**0.511 ± 0.009**[b] **P < 0.001**	0.391 ± 0.004 NS	0.398 ± 0.012 NS	0.383 ± 0.004 NS
2a	96	**0.844 ± 0.016** **P < 0.001**	**0.640 ± 0.005** **P < 0.001**	0.373 ± 0.013 NS	0.363 ± 0.019 NS	**0.529 ± 0.007** **P < 0.001**	0.401 ± 0.003 NS	0.376 ± 0.002 NS	0.384 ± 0.011 NS
2a-2	142	**0.676 ± 0.005** **P < 0.001**	**0.447 ± 0.005** **P < 0.001**	0.389 ± 0.014 NS	0.381 ± 0.012 NS	**0.584 ± 0.005** **P < 0.001**	0.401 ± 0.010 NS	0.331 ± 0.016 NS	0.391 ± 0.003 NS
2a-2a	184	**0.655 ± 0.019** **P < 0.001**	**0.426 ± 0.006** **P < 0.001**	0.408 ± 0.007 NS	0.402 ± 0.002 NS	**0.878 ± 0.007** **P < 0.001**	**0.660 ± 0.014** **P < 0.001**	0.352 ± 0.022 NS	0.380 ± 0.008 NS
2a-2b-3	257	**0.458 ± 0.021** **P < 0.001**	0.358 ± 0.002 NS	0.368 ± 0.007 NS	0.325 ± 0.009 NS	**0.954 ± 0.012** **P < 0.001**	**0.671 ± 0.009** **P < 0.001**	**0.507 ± 0.013** **P < 0.001**	0.335 ± 0.011 NS
MABs extract-free control	—	0.368 ± 0.005 (N = 12)				0.349 ± 0.011 (N = 6)			

[a]STHE-Bcl-2 parental cell line was inoculated subcutaneously into normal adult Syrian hamsters and after different periods of sc growth (96–257 days) variant TC lines were isolated from the tumor nodules, grown in vitro, and extracted. Extracts adjusted to 0.2 mg/1.0 ml of protein (undiluted) and diluted 1:2–1:8 were examined in parallel in modified sandwich ELISA with two MABs (anti-Bcl-2 and anti-Bax), each diluted 1:4,000; anti-mouse HRP-labeled rat antibodies were used for visualization of the ELISA test. Three wells of a 96-well plate used for each dose of cellular extract (undiluted or diluted), were pretreated with the capture MABs, and covered with the upper MABs (details in Materials and Methods).

[b]The mean OD values of each three wells (M ± m) and the Student's *t*-test were used for determination of *P*-values, that is, the significance of the differences between each sample and the mean OD level of control wells with extract-free MABs. The dashed cross lines demonstrate the direction and the border line between two alternative processes, that is of the gradual loss of Bcl-2 and activation of endogenous Bax in dynamics of in vivo STHE-Bcl-2 sc tumor growth and progression. NS, insignificant difference.

TABLE II. Dynamics of Bcl-2/Bax and Bax/Bcl-2 Ratios During In Vivo Experience of STHE-Bcl-2 Cells Under Conditions of In Vivo sc Growth and Progression

STHE-Bcl-2-[a] variant cell lines	In vivo experience (days)	Ratios	
		Bcl-2/Bax	Bax/Bcl-2
parental	0	3.29	0.30
2a	96	2.63	0.38
2a-2	142	1.30	0.76
2a-2a	184	0.54	1.84
2a-2b-3	257	0.14	6.76

[a]Undiluted extracts (0.2 mg/1.0 ml total protein) of STHE-Bcl-2 variant cell lines, parental and isolated from the sc growing tumor nodules at different times (in days) of their in vivo growth and progression, were examined in sandwich ELISA for the production of Bcl-2 and Bax using the corresponding MABs (anti-Bcl-2 and anti-Bax). Bcl-2/Bax and Bax/Bcl-2 ratios were determined in accordance to the OD data presented in Table I and the formula: mean values of OD (of Bcl-2, or Bax) minus mean values of OD of corresponding control wells treated with the same MABs, free of cell extracts, divided by the mean OD values (of Bax or Bcl-2) minus mean OD values of the corresponding extract-free control.

latter case, parental STHE-bcl-2 cell line and its four variant tumor cell lines, selected by chance from the same collection of the above 13 tumor cell lines were examined in the same sandwich ELISA, but the results were presented as the mean OD values ($M \pm m$), with indication of the levels of SE and the *P*-values of each measurement (in comparison with the mean OD values of corresponding controls). After 142–257 days of in vivo sc tumor growth, significantly lowered levels of Bcl-2 protein (up to its almost complete loss) could be determined in the STHE-Bcl-2 TC extracts challenged against anti-Bcl-2 MABs (Table I), while the initial low basic level of free Bax protein in the same cell extracts at the same time increased to a maximum. The latter data demonstrated the coincidence of two opposing processes in the same TC variants that is, the gradual in vivo downregulation of Bcl-2 and the alternative activation of Bax production.

The mean OD values (Table I) obtained in the study of five extracts of above STHE-Bcl-2 variant TC lines, challenged in parallel against anti-Bcl-2 and anti-Bax MABs, were used for the estimation of Bcl-2/Bax and of Bax/Bcl-2 ratios determined in undiluted extracts of TC samples in dynamics of in vivo sc tumor growth and progression (Table II). The data demonstrated that while Bcl-2 expression by STHE-Bcl-2 parental cells gradually decreased during their in vivo growth and the high initial Bcl-2/Bax index correspondingly gradually falls to the control level, activation of endogeneous Bax protein production in extracts of the same variants of in vivo experienced STHE-Bcl-2 cells resulted in simultaneously high increase of Bax/Bcl-2 ratio.

In a series of parallel experiments, we significantly extended our earlier observations [Dyakova et al., 2001] on the dynamics of acquisition of HP-phenotype by STHE and STHE-Bcl-2 cell lines at different time intervals after their iv dissemination. The summarized data (Table III) confirmed that acquisition of HP^+ phenotype by several descendant variants of the parental STHE cells was noted already at 5–15 days after iv inoculation, that is, its appearance was 10–20 times accelerated compared with acquisition of HP-phenotype at 100 ± 20 days by the same STHE (Bcl-2^-) cells grown sc. In contrast, all STHE-Bcl-2 lung-isolated TC variants remained HP^- during the entire time of their in vivo experience between 1 and 70 days after iv dissemination.

Unexpectedly, STHE TC (Bcl-2^-) in conditions of iv dissemination demonstrated immediate and extremely high Bax and Ras protein activation by all disseminated and lung-isolated STHE TC variants, beginning from the first day of in vivo experience (Fig. 4A). In most cases, it coincided with significantly increased production of HSP90/HSP70 and with the highly accelerated acquisition of HP-phenotype (black triangles) by several STHE cell variants at the period between 1 and 20 days after iv inoculation. Some decrease of Bax and Ras, followed with lowered levels of stress proteins production in the majority of STHE variant cell lines was noted in some TC variants at 15–20 days of their in vivo experience, while acquired expression of HP-phenotype remained stable (Fig. 4A). The low basal levels of endogenous Bcl-2 protein production by the parental STHE (Bcl-2^-) cells became even lower in almost all iv disseminated lung-isolated TC variants during the first 10 days after iv inoculation; in all cases, it coincided with the high activation of Bax protein production (Fig. 4A). The slightly increased levels of endogenous Bcl-2 protein production (or its restoration to basal level) were noted in some STHE cell variants between the 5th and 20th days.

Surprisingly, beginning from the first 2 days of in vivo experience of STHE-Bcl-2 cells under conditions of dissemination, a gradual decrease in initially high level of Bcl-2 was observed; it

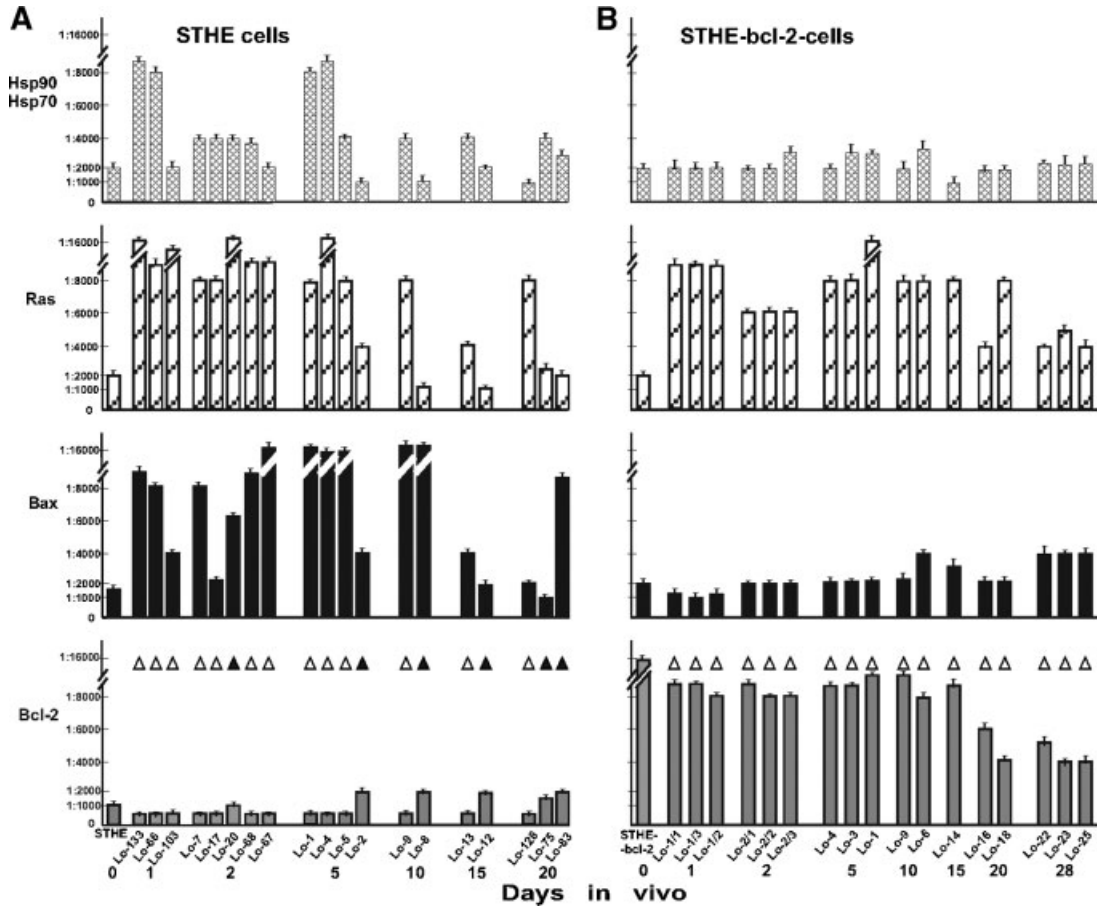

Fig. 4. **A**, **B**: Dynamics of Bcl-2, Bax, Ras, and HSP90/HSP70 production by iv disseminated STHE and STHE-Bcl-2 tumor cells and acquisition of HP-phenotype (sandwich ELISA data). Single cell suspensions of STHE cells (A) and STHE-Bcl-2 cells (B) were inoculated iv ($2.0–5.0 \times 10^6$/1.0 ml) into the retroorbital venous cavity of normal adult Syrian hamsters. At different times (1–20 or more days) after iv inoculation, the descendant variants of both parental cell lines were isolated from lung tissues (free from visible metastases) of individual animals, grown in vitro, and examined. The levels of Bcl-2, Bax, Ras, and HSP90/HSP70 production by the parental and descendant variants of tumor cell lines were tested in sandwich ELISA with the corresponding MABs diluted from 1:1,000–1:2,000 to 1:16,000–1:32,000 (details in Materials and Methods) and on expression of HP-phenotype. Designations: same as in Figure 3A,B.

was significantly lowered at the 20th and 28th day cell samples (Fig. 4B) and comparable with the loss of Bcl-2 in the same cells grown sc for 96 days (compare Figs. 3B and 4B). Thus, the rate of Bcl-2 loss from iv disseminated TC seems to be at least three times accelerated compared with its loss under conditions of sc growth of the same cells.

TABLE III. Dynamics of In Vivo Acquisition of HP-Phenotype by iv Disseminated Lung-Isolated STHE and STHE-Bcl-2 Cells

	Acquisition of HP-phenotype (in days) after iv inoculation							
Parental cells[a]	1	2	5	9–10	15	20	28–30	70
STHE	0/4[b]	0/5	1/5	2/3	5/6	4/6	n.d.	n.d.
STHE-Bcl-2	0/3	0/3	0/4	0/3	0/1	0/3	0/6	0/4

[a]Single cell suspensions of the parental STHE and STHE-Bcl-2 cell lines grown in vitro were inoculated ($2.0–5.0 \times 10^6$/1.0 ml) intravenously (iv) in normal Syrian hamsters. At the different time intervals after iv inoculation, the animals were sacrificed, tumor cells were isolated from the macroscopically normal lung tissues (free from visible metastases) of individual animals, grown in tissue culture, and examined in vitro on the expression of HP-phenotype (details in Materials and Methods).
[b]Numerator—number of lung-isolated TC variants that acquired the expression of HP-phenotype; denominator—number of TC variants examined.

In contrast to STHE cells, in iv disseminated STHE-Bcl-2 cells, production of Bax protein during the first 10 days in vivo remained suppressed at near the control level; it slightly increased in some cell samples examined between the 10th and 28th days, which coincided with the beginning of the parallel decrease in Bcl-2 protein production by these cells (Fig. 4B). Almost no increase in production of HSP90/HSP70 was registered during 1–28 days of in vivo experience of disseminated STHE-Bcl-2.

In contrast to Bax, high and immediate activation of endogenous Ras protein production was induced in all disseminated cell variants of both, STHE and STHE-Bcl-2 cell lines beginning from the first day of in vivo experience of these cells; it slightly decreased during the 15th–28th day observation periods (Fig. 4A,B). The relatively high initial activation of Ras production in sc grown STHE (Bcl-2+/−) cells later also gradually decreased (Fig. 3A,B).

Thus, during in vivo experience of STHE and STHE-Bcl-2 tumor cell lines (Figs. 3A,B and 4A,B), the initially low basal Bcl-2 level of STHE cells correlated with immediate and high activation of Bax, Ras, and HSP90/HSP70 and accelerated acquisition of HP-phenotype, while the high initial expression of Bcl-2 protein production by STHE-bcl-2 cells corresponded to suppressed production of Bax and HSP90/HSP70 and the delayed tumor progression (Figs. 3A,B and 4A,B, Tables I, II, and IV). This picture changes at the late progression stage of STHE-bcl-2 cell line after 142–184 days of in vivo sc tumor growth when the almost complete loss of Bcl-2 correlated with the simultaneous high activation of Bax production (Fig. 3B).

DISCUSSION

The data presented in the study seem to contradict the following widely accepted points of view in this field: (1) the better in vivo survival of Bcl-2^+ TC is usually considered as a condition a priori promoting tumor progression. However, if it is so, it should apparently decrease the level of the host environmental selection pressure against TC and thus, in opposite, may delay tumor progression; earlier such delay was demonstrated in our studies [Deichman et al., 1998]; (2) in vivo host recognized tumor cells are usually considered as a targets of different growth and death factors produced by the host effector cells [rev. in Dunn et al., 2002]. The responses of TC to these signals are mainly registered as better or worse survival, or apoptosis. The possible ability and readiness of TC to respond to the host environmental stress by immediate primary reactions, expressed in activation, or a suppression of a set of endogenous death-signaling proteins as yet were not regarded.

To our knowledge, this is the first study describing the primary in vivo reactions of Bcl-2-protected and not protected TC to the stress signals of the host normal cellular environment, expressed in immediate activation (or suppression) of several endogenous proapoptotic proteins, such as Bax, Ras and HSP90/HSP70. The stress conditions of the local versus disseminated tumor growth, examined in these model experiments were almost physiological

TABLE IV. In Vivo Activation of Endogenous Death-Signaling Proteins in STHE Cells, Their Inhibition in STHE-Bcl-2 Cells, and the Rates of Tumor Progression (Summarized Data)

Cells in vivo experience	STHE (Bcl-2^-) cells	STHE (Bcl-2^+) cells		
Apoptosis	↑	↓		
Survival	↓	↑		
Expression of				
Bcl-2	–	+++	→*	–
Bax	+++	–	→*	+++
HSP90	+++	–	→*	+++
HSP70	+++	–	→*	+++
Ras	+++	+++	→*	++
Rates of tumor progression →	↓ Accelerated	↓ Delayed	→*	↓ Accelerated

Designations: ↓↑- up- and downregulation of corresponding functions; +++ or − activation or suppression of the corresponding proteins in vivo in STHE (Bcl-2^-) and STHE (Bcl-2^+) cells; *—transition in dynamics of tumor progression in vivo.

and as near, as possible to conditions of the natural in vivo tumor progression. The use in these in vivo selection studies of non-immunogenic STHE cells, as a parental ones, apparently excluded involvement of the specific (adaptive) immune antitumor reactions from the given host-tumor cells interactions and demonstrated TC primary reactions to the host normal cellular environment.

The results of the present study demonstrate the following new findings:

(1) **Great qualitative differences between Bcl-2-protected and unprotected STHE cells in their primary in vivo reactions to the contact interaction with the host normal cellular environment, especially evident in higher stress conditions of dissemination (Figs. 3A,B and 4A,B)**. Surprizingly, STHE cells not protected by Bcl-2 in the latter conditions demonstrated immediate (within a few hours after iv inoculation) reaction expressed in activation of proapoptotic Bax, Ras, and HSP90/HSP70 stress protein production, maintained in vivo at a high level for about 10 days, coincided in time with accelerated acquisition of HP-phenotype by some of STHE (Bcl-2$^-$) TC variants (Fig. 4A). Apparently, activation of Bax production (as well as of HSP90/HSP70 and Ras) represents the normal immediate proapoptotic reaction of TrC not protected by Bcl-2 to the stress signaling of the host cellular environment. In time, it exactly coincided with the in vivo mass death of iv-inoculated TC demonstrated years ago [Fidler, 1970; Poste and Fidler, 1980; Price et al., 1984, 1986]. In contrast, STHE-Bcl-2 cells under the same conditions demonstrated no immediate activation of Bax and of stress protein production, as well as the highly delayed acquisition of HP-phenotype and tumor progression. It follows, that besides the antiapoptotic activity, STHE-Bcl-2 cells appear to be capable of suppressing immediate activation of endogenous Bax and thus of the normal proapoptotic reaction of unprotected STHE TrC to the host cellular environment (Fig. 4A,B).

(2) **The unexpected gradual in vivo downregulation of Bcl-2 production by STHE-Bcl-2 cells during tumor progression up to almost complete loss.** This process, slow under conditions of sc tumor growth, was about three times accelerated under higher stress conditions of TC iv dissemination. The mechanisms determining the gradual downregulation of Bcl-2 production in STHE-bcl-2 tumor cells in both in vivo regimes is unclear, but it suggests at least three different possibilities: [A] the constant environmental stress-signaling of the host normal cells, at last leading to Bcl-2 suppression and simultaneous activation of endogenous Bax; [B]. the formation of heterodimers between Bcl-2 and Bax modulating functional cellular antiapoptotic and proapoptotic activities of the corresponding tumor cells; [C] the Bax-activating stress reaction of wt p53.

As shown earlier, due to heterodimerization the excess of Bcl-2 protein rendered low levels of free proapoptotic Bax and expressed antiapoptotic activity, while high levels of pro apoptoic Bax were inhibiting Bcl-2 antiapoptotic activity [Oltvai et al., 1993; Yin et al., 1994; Hsu and Youle, 1997; Otter et al., 1998; rev in Adams and Cory, 1998; Hirotani et al., 1999; Yin, 2006; and in van Delft and Huang, 2006]. This is reminiscent of the following two opposite situations (Fig. 4A,B) observed in our present studies with disseminated STHE and STHE-Bcl-2 cells, when: (1) high initial level of Bcl-2 and the low basal Bax of STHE-bcl-2 cells creates disbalance between Bcl-2 and Bax in favor of antiapoptotic Bcl-2 (Fig. 4B); correspondingly, high Bcl-2/Bax ratios and antiapoptotic activity are characteristic for these cells (Table I); (2) immediate activation and high production of Bax in Bcl-2$^-$ STHE cells creates opposite disbalance between the high level of activated Bax and the low basal level of Bcl-2; correspondingly the Bax/Bcl-2 ratio in these cells changes in favor of proapoptotic Bax (Fig. 4A and Tables I and II). In both of these situations, the proportion of Bcl-2-Bax equimolar heterodimeres in TC extracts should be relatively low. It may be suggested that in ELISA, the corresponding MABs apparently determine mainly the free forms of these proteins, while the heterodimerized forms of Bcl-2 and Bax may be less accessible for these MABs.

A strong positive correlation between p53 overexpression and Bax expression levels was recently recorded in patients with the pancreatic adenocarcinoma [Magistrelli et al., 2006].

The normally low level of wt p53 as a rule increases following different stress signals and DNA damage of TC. Earlier shown activation of endogenous Bax (downstream to wt p53) at the protein and mRNA levels may apparently also be involved in downregulation of Bcl-2 production [Haldar et al., 1994; Miyashita and Reed, 1995; Zornig et al., 2001].

Recently, some data in favor of the possible restriction by Bcl-2 of the mutated p53 appearance in STHE-Bcl-2 cells, as a condition leading to the delay of tumor progression were presented [Gurova et al., 2002; rev in Gurova and Gudkov, 2003]. The data of our present study favor a different explanation of this phenomenon and consider the delay of tumor progression as a consequence of the demonstrated ability of Bcl-2 in STHE-bcl-2 cells to suppress activation of Bax and its proapoptotic activity. Moreover, the earlier shown opposite effect, that is, acceleration of STHE tumor progression under conditions of dissemination, that is, of higher environmental stress, as shown in the present study correlated with the immediate activation of endogenous proapoptotic Bax, downregulation of Bcl-2 and acceleration of tumor progression (Fig. 4A and Table IV). This allows to connect the mechanisms of the in vivo delayed or accelerated tumor progression correspondingly with the suppression, or activation of proapoptotic Bax. In vivo sublethal damage of STHE cell DNA by activated endogenous Bax, and the repair of the damaged cell proteins by immediately activated HSP90/HSP70 chaperones, may be the prerequisites for the accelerated appearance of genetically altered variants of TC and the subsequent selective competition of the variety of phenotypically diverse TC variants, including those of HP-phenotype. This suggestion is in line with the recently shown accelerated development of lymphoma in Bax transgenic mice, inhibited by Bcl-2 and associated with aneuploidy and chromosome instability [Luke et al., 2003].

(3) **The alternative character of Bcl-2 and Bax proteins production by TC and their double role in tumor progression.** The dynamic relationships between Bcl-2 and Bax production by STHE-Bcl-2 tumor cell variants examined at the level of individual cell lines during their in vivo progression (up to 257 days of sc growth), demonstrate the alternative character and the mutual dependence of the expression of these two proteins (Figs. 3A,B and 4A,B, Tables I, II, and IV). As in the case of Bax immediate in vivo activation in disseminated STHE (Bcl-2$^-$) cells, Bax activation in the late progression stages of STHE-bcl-2 cells also took place on the background of completely suppressed Bcl-2 protein (Fig. 3B).

Apparently, Bcl-2 and Bax both play double and opposite roles in tumor development and progression. The delayed tumor progression of Bcl-2-producing cells is apparently connected not only with Bcl-2 direct antiapoptotic activity/that is, better in vivo survival of TC and the lower host selection pressure/, but, particularly, with Bcl-2 Bax-suppressing activity, thus preventing its proapoptotic DNA damaging activity. In contrast, acceleration of tumor progression of Bax-producing TC is apparently associated with its direct proapoptotic activity and, indirectly, with the suppression of Bcl-2 prosurvival activity. Correspondingly, the alternative expression of either Bcl-2-antiapoptotic or of Bax-proapoptotic cellular programs may apparently significantly narrow, or highly extend the field of the in vivo natural selection of the malignant TC variants and thus influence the rates of tumor progression.

(4) **Regular in vivo transition of antiapoptotic cell program of STHE-Bcl-2 cells, responsible for the delay of tumor progression, to Bcl-2 downregulation and alternative activation of proapoptotic Bax program, accelerating the process of tumor progression.** The study demonstrated (Figs. 3B and Table IV) that in vivo at the later stages of sc tumor growth after the almost complete loss of Bcl-2 from STHE-Bcl-2 cells the rate of tumor progression regularly change from initially delayed to accelerated, apparently due to the transit of the Bcl-2-antiapoptotic cell program to the alternative Bax-proapoptotic one (summarized in Table IV).

(5) **Immediate in vivo activation of HSP90/HSP70 stress proteins production following activation of Bax protein production.** As shown (Fig. 4A,B), immediate increase of HSP90/HSP70 production follows the immediate in vivo

activation of endogenous Bax in STHE TC not protected by Bcl-2. While the majority of in vivo disseminated unprotected TC die during the first 12–96 h [Fidler, 1970; Poste and Fidler, 1980; Price et al., 1984, 1986], a few TC would be rescued and recover, possibly due to the chaperone activity of stress proteins [reviewed by Jolly and Morimoto, 2000]. In contrast, the depletion of stress proteins promotes tumor specific death program, which is independent of caspases and bypasses Bcl-2 [Nylandsted et al., 2000].

(6) **Immediate in vivo activation of endogenous Ras protein production in both STHE (Bcl-2+/−) cell lines.** Apparently, immediate in vivo activation of Ras also represents earlier unknown normal primary in vivo reaction of STHE and STHE-Bcl-2 TrC to the host cellular environment (Fig. 4A,B). However, in contrast to Bax, immediate activation of Ras was equally highly expressed in both, STHE (Bcl-2+/−) parental cell lines. This apparently means that the host environmental signals leading to activation of endogenous Ras, in contrast to Bax activation, are not controlled by Bcl-2. Earlier, we demonstrated that transfection by N-ras or by Ha-ras of STHE cells leads to almost complete transient suppression of TC catalase activity and, correspondingly, to lowered antioxidant activity of these cells [Deichman et al., 1996; Kashkina et al., 2004]. It seems likely that immediate in vivo activation of proapoptotic Bax and of catalase-suppressing Ras in TC not protected by Bcl-2 are possibly both relevant to the earlier described mass in vivo death (mass suicide?) of TC observed during the first hours and days after iv inoculation of ^{125}IuDR-labeled TC [Fidler, 1970; Poste and Fidler, 1980; Price et al., 1984, 1986]. The immediate in vivo activation of endogenous death-signaling Bax and Ras (Fig. 4A), as a primary response of unprotected TC to the stress signals of the normal cellular environment may represent an essential part of the host antitumor strategy leading to activation of the suicide program in the majority of TC, but as well, to selection of the rare and more resistant TC variants.

(7) **The suggested mechanisms of the delay of tumor progression may elaborate some new approaches for human cancer treatment directed towards inhibition of tumor progression.** Recently, the quite opposite approach was used in order to suppress the growth of some TC lines in vivo and in vitro with the use of a small-molecule inhibitor of antiapoptotic Bcl-2 production, leading to regression of some solid tumors and lymphoma [Oltersdorf et al., 2005]. It remained unclear, if the treatment of TC with ABT-737 inhibitor not only suppressed Bcl-2 protein production, but also, led to the Bax activation. In the latter case, the cytotoxicity of the inhibitor used for the treatment of the target TC would apparently greatly increase due to activated Bax.

In general, the comparison of the in vivo dynamic behavior of STHE (Bcl-2+/−) cells suggests that high initial expression in these cells of either Bcl-2, or Bax may represent two alternative cell programs, which lead to the opposite consequences for in vivo tumor progression corresponding to either the delay or acceleration of this process. The summarized results of the study are illustrated in Table IV demonstrating the coincidence of the immediate activation of several endogenous death-signaling proteins in STHE cells, and particularly of Bax, with their accelerated in vivo progression. In contrast, initial high expression of Bcl-2 and the suppression of proapoptotic Bax production in STHE-Bcl-2 cells corresponded to the delay of in vivo tumor progression, which later, in the case of Bcl-2 loss, would inevitably transit into activation of Bax and acceleration of tumor progression.

In conclusion, the data presented demonstrate that during in vivo development of TrC, not protected against apoptosis by Bcl-2, there is a relatively short initial period of about 100 (±20) days for the locally growing tumors and a much shorter (few days) period for disseminated TC, when the rare sublethaly damaged cells, would apparently repair DNA, begin to proliferate, and undergo natural selection of some genetically unstable TC variants possessing selective advantages in vivo. High acceleration of tumor progression under conditions of dissemination as compared with the local tumor growth suggests that this seemingly shortest step of in vivo tumor development apparently represents the period when TC are most vulnerable for the environmental stress-induced DNA damage and the subsequent

phenotypic changes of TC. As shown in this study, the immediate reaction of unprotected TrC to normal cellular environment expressed in activation of proapoptotic Bax, Ras, and HSP90/HSP70 production and the early appearance of HP-phenotype-expressing variants among disseminated TC develop synchronously with accelerated tumor progression. In contrast, the antiapoptotic and Bax-suppressing activities of Bcl-2-expressing TrC apparently represent another cell program responsible for extinguished environmental stress signals, decreased selective pressure of the host against these cells and significantly delayed tumor progression. Thus, in accordance with the experimental model examined in this study, tumor progression in vivo begins immediately in contact interaction of TrC with the host normal cellular environment and proceeds depending on: (a) the initial anti- or pro-apoptotic programs of TrC, responsible for either its delay or acceleration, and (b) the regular in vivo transition of the initial Bcl-2 antiapoptotic cells program into the Bax proapoptotic program, apparently followed "on half of the way" with the corresponding change in the rate of tumor progression from delay towards acceleration. The hypothesis predicts that process of tumor progression in vivo can be regulated by manipulation of the anti- and pro-apoptotic activities of tumor cells.

ACKNOWLEDGMENTS

The authors greatly appreciate the helpful discussions with Professor Garry Abelev, the help of Elvira Uvarova in tissue culture work and of Tatiana Ivantsova and of Andrey Komelkov with the WB assays. This study was supported by grants Nos. 99-04-48358a, 02-04-48232a, and 05-04-49439a of the Russian Foundation for Basic Research.

REFERENCES

Adams JM, Cory S. 1998. The Bcl-2 protein family: Arbiters of cell survival. Science 281(5381):1322–1326.

Bauer G. 1996. Elimination of transformed cells by normal cells: A novel concept for the control of carcinogenesis. Histol Histopathol 11:237–255.

de La Coste A, Mignon A, Fabre M, Gilbert E, Porteu A, Van Dyke T, Kahn A, Perret C. 1999. Paradoxical inhibition of c-myc-induced carcinogenesis by Bcl-2 in transgenic mice. Cancer Res 59(19):5017–5022.

Deichman GI. 1988. Natural host resistance and in vivo selection of malignant tumor cells. Cancer Surveys 7(4): 675–690.

Deichman GI. 2000. Natural selection and early changes of phenotype of tumor cells in vivo: acquisition of new defense mechanisms. Biochemistry (Mosc). 65(1): 78–94.

Deichman GI. 2002. Early phenotypic changes of in vitro transformed cells during in vivo progression: Possible role of the host innate immunity. In Sem Cancer Biol 12(4):317–326.

Deichman GI, Vendrov EL. 1986. Characteristics of in vitro transformed cells essential for their in vivo survival, selection and metastasizing activity. Int J Cancer 37: 401–409.

Deichman GI, Kluchareva TE, Matveeva VA, Kushlinsky NE, Bassalyk LS, Vendrov EL. 1989. Clustering of discrete cell properties essential for tumorigenicity and metastasis. I. Studies of Syrian hamster embryo fibroblasts spontaneously transformed in vitro. Int J Cancer 44:904–907.

Deichman GI, Gurova KV, Dyakova NA. 1994 . Accumulation in the "normal" Lungs of live tumorigenic cells during subcutaneous growth of weakly and highly metastasizing tumors and their susceptibility to growth regulation by normal fibroblasts. Int J Cancer 59:530–537.

Deichman GI, Kashkina LM, Mezenina OA, Gorojanskaya EG, Nikiforov MA, Gudkov AV, Dyakova NA, Komelkov AV, Prilutskaya MO, Tatosyan AG. 1996. Mechanisms of unusually high antioxidant activity of RSV-SR-transformed cells and of its suppression by activated $p21^{ras}$. Int J Cancer 66:747–752.

Deichman GI, Matveeva VA, Kashkina LM, Dyakova NA, Uvarova EN, Nikiforov MA, Gudkov AV. 1998. Cell transforming genes and tumor progression: In vivo unified phenotypic secondary cell changes. Int J Cancer 75(2):277–283.

Dotto GP, Weinberg RA, Ariza A. 1988. Malignant transformation of mouse primary keratinocytes by Harvey sarcoma virus and its modulation by surrounding normal cells. PNAS 85:6389–6393.

Dunn GP, Bruce AT, Ikeda H, Old LJ, Schreiber RD. 2002. Cancer immunoediting: From immuno-surveillance to tumor escape. Nature immunology 3:991–998.

Dyakova NA, Kashkina LM, Matveeva VA, Uvarova EN, Deichman GI. 2001. Dynamics of the natural selection of tumor cells of $(H_2O_2{}^{CA} + PGE^{S})$ phenotype in conditions of subcutaneous growth and dissemination. Dokl Biol Sci 378:283–286.

Fidler IJ. 1970. Metastasis: Quantitative analysis of distribution and fate of tumor emboli labeled with ^{125}I-5-Iodo-2′-deoxyuridine. J Natl Cancer Inst 45:733–782.

Fidler IJ. 2003. The pathogenesis of cancer metastasis: The 'seed and soil' hypothesis revisited. Nat Rev Cancer 3:453–458.

Graeber TG, Osmanian C, Jacks T, Housman DE, Koch CJ, Lowe SW, Giaccia AJ. 1996. Hypoxia-mediated selection of cells with diminished apoptotic potential in solid tumours. Nature 379:88–91.

Gurova KV, Gudkov AV. 2003. Paradoxical role of apoptosis in tumor progression. J Cell Biochem 88(1):128–137.

Gurova KV, Kwek SS, Koman IE, Komarov AP, Kandel E, Nikiforov MA, Gudkov AV. 2002 . Apoptosis inhibitor as a suppressor of tumor progression: Expression of Bcl-2 eliminates selective advantages for p53-deficient cells in the tumor. Cancer Biol Ther 1:39–44.

Haldar S, Negrini M, Monne M, Sabbioni S, Croce CM. 1994. Down-regulation of bcl-2 by p53 in breast cancer cells. Cancer Res 54(8):2095–2097.

Hanahan D, Weinberg RA. 2000. The hallmarks of cancer. Cell 100:57–70.

Hanusch J, Schwieger A, Sers C, Schafer R, Bauer G. 2000. Oncogenic transformation increases the sensitivity for apoptosis induction by inhibitors of macromolecular synthesis. Int J Oncol 17:89–95.

Heigold S, Sers C, Bechtel W, Ivanovas B, Schafer R, Bauer G. 2002. Nitric oxide mediates apoptosis induction selectively in transformed fibroblasts compared to non-transformed fibroblasts. Carcinogenesis 23(6):929–941.

Hirotani M, Zhang Y, Fujita N, Naito M, Tsuruo T. 1999. NH_2-terminal BH4 domain of Bcl-2 is functional for heterodimerization with Bax and inhibition of apoptosis. J Biol Chem 274(29):20415–20420.

Howard JC, Li Q, Chu W, Zochodne B, Kapoor M, Ung Y, Rosen K, Ben-David Y. 2001. Bcl-2 expression in F-MuLV-induced erythroleukemias: A role for the anti-apoptotic action of Bcl-2 during tumor progression. Oncogene 20(18):2291–2300.

Hsu YT, Youle RJ. 1997. Nonionic detergents induce dimerization among members of the Bcl-2 family. JBC 272:13829–13834.

Ionov Y, Yamamoto H, Krajewski S, Reed JC, Perucho M. 2000. Mutational inactivation of the proapoptotic gene Bax confers selective advantage during tumor clonal evolution. PNAS 97(20):10872–10877.

Jolly C, Morimoto RI. 2000. Role of the heat shock response and molecular chaperones in oncogenesis and cell death. J Natl Cancer Inst 92(19):1564–1572.

Kashkina LM, Matveeva VA, Dyakova NA, Uvarova EN, Deichman GI. 2004. Acquisition of HP phenotype and alterations in the activity of catalase of in vitro transformed cells of different origin in the dynamics of in vivo tumor progression. Dokl Biol Sci 394:94–97.

Klein G. 2002. Introduction: Genetic and epigenetic contribution to tumor evolution. Sem Cancer Biol 12:327–330.

Klein G. 2004. Cancer, apoptosis, and nonimmune surveillance. Cell Death Different 11:13–17.

Klein G, Klein E. 2005. Surveillance against tumors—Is it mainly immunological? Immunol Lett 100(1):29–33.

Kluchareva TE, Matveeva VA, Kushlinsky NE. 1992. In vitro bioassay for type E prostaglandins based on their NK immunodepressing activity. Imm Lett 33(3):239–246.

Knudson CM, Korsmeyer SJ. 1997. Bcl-2 and Bax function independently to regulate cell death. Nat Genet 16(4): 358–363.

Knudson CM, Johnson GM, Lin Y, Korsmeyer SJ. 2001. Bax accelerates tumorigenesis in p53-deficient mice. Cancer Res 61(2):659–665.

Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685.

Luke JJ, van der Wetering CJ, Knudson CM. 2003. Lymphoma development in Bax transgenic mice is inhibited by Bcl-2 and associated with chromosomal instability. Cell Death Different 6:540–548.

Magistrelli P, Coppola R, Tonini G, Vincenzi B, Santini D, Borzomati D, Vecchio F, Valeri S, Castri F, Antinori A, Nuzzo G, Caraglia M, Picciocchi A. 2006. Apoptotic index or a combination of Bax/Bcl-2 expression correlate with survival after resection of pancreatic adenocarcinoma. J Cell Biochem 97(1):98–108.

Miyashita T, Reed JC. 1995. Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. Cell 80(2):293–299.

Nowell PS. 1986. The clonal evolution of tumor cell subpopulations. Science 194:23–28.

Nylandsted J, Rohde M, Brand K, Bastholm L, Elling F, Jaattela M. 2000. Selective depletion of heat shock protein 70 Hsp70 activates a tumor-specific death program that is independent of caspases and bypasses Bcl-2. PNAS 97(14):7871–7876.

Oltersdorf T, Elmore SW, Shoemaker AR, Armstrong RC, Augeri DJ, Belli BA, Bruncko M, Deckwerth TL, Dinges J, Hajduk PJ, Joseph MK, Kitada S, Korsmeyer SJ, Kunzer AR, Letai A, Li C, Mitten MJ, Nettesheim DG, Ng S, Nimmer PM, O'Connor JM, Oleksijew A, Petros AM, Reed JC, Shen W, Tahir SK, Thompson CB, Tomaselli KJ, Wang B, Wendt MD, Zhang H, Fesik SW, Rosenberg SH. 2005. An inhibitor of Bcl-2 family proteins induces regression of solid tumours. Nature 435:677–681.

Oltvai ZN, Milliman CL, Korsmeyer SJ. 1993. Bcl-2 heterodimerizes in vivo with a conserved homolog Bax, that accelerates programmed cell death. Cell 74(4):609–619.

Otter I, Conus S, Ravn U, Rager M, Olivier R, Monney L, Fabbro D, Borner C. 1998. The binding properties and biological activities of Bcl-2 and Bax in cells exposed to apoptotic stimuli. J Biol Chem 273(11):6110–6120.

Poste G, Fidler IJ. 1980. The pathogenesis of cancer metastasis. Nature 283:139–146.

Price JE, Carr D, Tarin D. 1984. Spontaneous and induced metastases of naturally occurring tumors in mice: Analysis of cell shedding into the blood. J Natl Cancer Inst 73:1319–1326.

Price JE, Aukerman SL, Fidler IJ. 1986. Evidence that the process of murine melanoma metastasis is sequential and selective and contains stochastic elements. Cancer Res 46:5172–5178.

Roskelley CD, Bissell M. 2002. The dominance of microenvironment in breast and ovarian cancer. Sem Cancer Biol 12:97–104.

Sierra A, Castellsague X, Escobedo A, Lloveras B, Garcia-Ramirez M, Moreno A, Fabra A. 2000. Bcl-2 with loss of apoptosis allows accumulation of genetic alterations: A pathway to metastatic progression in human breast cancer. Int J Cancer 89(2):142–147.

Vail ME, Pierce RH, Fausto N. 2001. Bcl-2 delays and alters hepatic carcinogenesis induced by transforming growth factor alpha. Cancer Res 61(2):594–601.

Valdes J, Garcia E, Llorente M, Mendes E. 2003. Innovative approach to low-level gluten determination in foods using a novel sandwich enzyme-liked immunosorbent assay protocol. Eur J Gastroenterol Hepatol 15(5):465–474.

van Delft MF, Huang DC. 2006. How the Bcl-2 family of proteins interact to regulate apoptosis. Cell Res 16(2): 2003–2013.

van Slooten HJ, van de Vijver MJ, van de Velde CJ, van Dierendonck JH. 1998. Loss of Bcl-2 in invasive breast cancer is associated with high rates of cell death, but also with increased proliferative activity. Br J Cancer 77(5): 789–796.

Weinberg R. 1989. Oncogenes, anti-oncogenes and the molecular bases of multistep carcinogenesis. Cancer Res 49:3713–3721.

Witz IP, Sagi-Assif O, Ran M. 1996. The shaping of the malignant phenotype—An interplay between cellular characteristics and microenvironmental factors. In: Yefenof E, Scheuermann RH, editors. Premalignancy and Tumor Dormancy. Heidelberg: RG Landes Company, Springer-Verlag.

Yin XM. 2006. Bid, a BH3-only multifunctional molecule, is at the cross road of life and death. Gene 369:7–19.

Yin XM, Oltvai ZN, Korsmeyer SL. 1994. BH1 and BH2 domains of Bcl-2 are required for inhibition of apoptosis and heterodimerization with Bax. Nature 369(6478): 321–323.

Young MR, Knies S. 1984. Prostaglandin E production by Lewis lung carcinoma: Mechanism for tumor establishment in vivo. J Natl Cancer Inst 72:919–922.

Zornig M, Hueber A, Baum W, Evan G. 2001. Apoptosis regulators and their role in tumorigenesis. Biochim Biophys Acta 1551(2):F1–F37.