Activin A-induced apoptosis is suppressed by BCL-2

Takeyoshi Koseki[a], Kenji Yamato[a,b], Stanislaw Krajewski[c], John C. Reed[c], Yoshihide Tsujimoto[d], Tatsuji Nishihara[a,*]

[a]*Department of Oral Science, The National Institute of Health, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162, Japan*
[b]*Department of Molecular and Cellular Oncology/Microbiology, Tokyo Medical and Dental University, Faculty of Dentistry, 1-5-45, Yushima, Bunkyou-ku, Tokyo 113, Japan*
[c]*La Jolla Cancer Research Foundation, Cancer Research Center, 10901 North Torrey Pines Road, 419 North Building, La Jolla, CA 92037, USA*
[d]*Biomedical Research Center, Osaka University Medical School, 2-2, Yamadaoka, Suita, Osaka, 565, Japan*

Received 2 October 1995; revised version received 30 October 1995

Abstract Activin A, a member of TGFβ superfamily has various activities including induction of apoptosis in mammalian cells. However, it remains unknown how activin A induces cell death. To clarify this, we investigated the expression of BCL-2 and BAX, and the effect of BCL-2 overexpression on activin A-induced apoptosis in B cell hybridoma cell lines. The activin A-sensitive cell lines expressed BAX but not BCL-2 and that activin A did not increase BAX levels. Overexpression of human BCL-2 suppressed activin A-induced apoptosis in these cells. Thus, activin A has been shown to induce apoptosis by a BCL-2-inhibitable mechanism without activating BAX.

Key words: Activin A; Apoptosis; BAX; BCL-2; Programmed cell death

1. Introduction

Activin A was originally isolated as a factor stimulating secretion of follicle-stimulating hormone (FSH) from anterior pituitary cells [1]. It possesses various in vitro activities such as enhancement of neuronal survival [2], induction of erythroid differentiation [3] and mesoderm formation during amphibian embryonic development [4,5]. Recently, we reported that activin A induces apoptotic cell death of some myelomas, and that TGFβ shows no cytotoxic effect on these cells [6]. Activins bind to cell-surface molecules of 50–55 kDa and 70–75 kDa known as activin receptors type I (ActR I) [7] and type II (ActR II) [8]. These receptors are members of a newly-defined family of transmembrane serine/threonine kinase, which includes the *daf-1* gene product of *Caenorhabditis elegans* and the type II TGFβ receptor [9]. Activin A may mediate a variety of activities through different combinations of ActR subtypes or isotypes.

We previously reported that activin A derived from activated macrophages induced apoptotic cell death in B lineage cells [10], suggesting the possible role of activin A in B cell immunoregulation. Apoptosis is a self-killing mechanism with morphological and biochemical characteristics and contributes to the structural reorganization during embryonic development, clonal selection in the immune system and abortion of cells with damaged DNA [11]. The *BCL-2* product is a 26-kDa protein localized to outer-membranes of nuclei, mitochondria and endoplasmic reticula [12]. The ability of *BCL-2* to enhance the survival of hematopoietic cells is related to its ability to block apoptosis caused by growth factor deprivation [13]. However, some types of apoptosis cannot be blocked by expression of exogenous *BCL-2* [14]. Recently, genes related to *BCL-2* have been cloned and form the *BCL-2* family [15]. Oltvai et al. reported that *BAX* product, a member of the *BCL-2* family, antagonizes the anti-apoptotic activity of *BCL-2* by forming a heterodimer with BCL-2 [16]. Failure of *BCL-2* to block apoptosis may be explained by involvement of other members of the *BCL-2* family in the regulation of apoptosis in certain types of cells or apoptotic stimuli. In this study, we investigated the mechanism of activin A-induced B cell apoptosis by analyzing *BCL-2* and *BAX* expression and the effect of *BCL-2* overexpression on activin A-induced B cell death. We show here that induction of high levels of human *BCL-2* blocks activin A-induced apoptosis and that activin A does not enhance expression of *BAX*.

2. Materials and methods

2.1. Cell culture and plasmid

Establishment and characterization of mouse hybridoma HS-5 and HS-7 cell lines were described previously [10]. HS-72 is a subclone of HS-7 cell line sensitive to activin A-induced cell death. Mouse leukemic M1 and human monoblastic U937 cell lines were obtained from RIKEN Cell Bank and Japanese Cancer Research Resource Bank, respectively. HS-5, HS-72 and M1 cells were cultured in Iscove's modified Dulbecco's medium (IMDM; GIBCO Laboratories, Grand Island, NY) and U937 cells were maintained in RPMI 1640 (GIBCO Laboratories), both containing 10% heat-inactivated fetal calf serum (FCS), 100 μg/ml streptomycin and 100 U/ml penicillin. Construction of *BCL-2* expression plasmid pCΔj-bcl-2 and control plasmid pCΔj-SV2 was reported [17]. Cells were transfected with plasmid by electroporation using a Electroporator II (Invitrogen, San Diego, CA) at 200 V, 1000 μF and then selected by cultivation with G418 (450 μg/ml). Single-cell clones were obtained by limiting dilution.

2.2. MTT assay

Cells seeded at a density of 2×10^5/ml in 96-well plates were incubated with IMDM containing 5% FCS and antibiotics in the presence of various concentrations of activin A at 37°C and then examined for cell viability by colorimetric assay with MTT (3-[4,5-dimethylthiazol2-yl]-2,5-diphenyltetrazolium bromide, Sigma Chemical Co., St. Louis, MO) [6]. Absorbance was determined at a wave length of 570 nm with background subtraction at 620 nm.

2.3. Detection of apoptotic cells

To detect apoptotic nuclei, the cells were suspended in hypotonic solution (3.4 mM sodium citrate, 0.1% Triton X-100, 0.1 mM EDTA, 1 mM Tris), stained with 5 μg/ml of propidium iodide and analyzed by a FACScan (Becton Dickinson Immunocytometry Systems, San Jose, CA) [18]. In DNA fragmentation assay, the cells (5×10^6) were treated by proteinase K at 37°C and cellular DNA was extracted by phenol/chloroform [19], electrophoresed in a 2% agarose gel and stained with ethidium bromide.

*Corresponding author. Fax: (81) (3) 5285-1172.

SSDI 0014-5793(95)01290-7

2.4. Immunoblot analysis

A monoclonal antibody specific to human BCL-2 (clone 124) was purchased from Boehringer Mannheim (Mannheim, Germany). Rabbit anti-mouse BCL-2 and anti-mouse BAX sera were raised against synthetic peptides as described previously [20]. The cells were lysed in ice-cold lysis buffer (40 mM Tris-HCl, pH 7.4, 140 mM NaCl, 1% NP-40) and 20 μg of protein extracts were electrophoresed in 12.5% SDS-polyacrylamide gels, electroblotted on nitrocellulose filters and reacted with primary antibodies. Signals were detected by a ECL detection kit (Amersham, Buckinghamshire, UK).

3. Results

Expression of endogenous *BCL-2* was studied in mouse B cell hybridoma cell lines (HS-5, HS-72) susceptible to activin A-induced apoptosis. Immunoblot analysis revealed that HS-5 and HS-72 clones expressed negligible levels of endogenous mouse *BCL-2* as compared with M1 mouse myeloblastic cell line (Fig. 1). To test if activin A-triggered apoptotic process can be inhibited by BCL-2, HS-5 and HS-72 cell lines were transfected with human *BCL-2* expression plasmid (pCΔj-bcl-2) or control plasmid (pCΔj-SV2) and stable transfectants were selected by culturing with G418. Two clones (HS-5B-3, HS-5B-6) from HS-5 and one clone (HS-72B-16) from HS-7 were found to express high levels of human *BCL-2* as compared with U937 human monoblastic cell line and used for further analyses (Fig. 1). Control plasmid-transfected HS-5S-2 clone from HS-5 and HS-72S-4 clone from HS-72 were used as controls.

The cytotoxic effect of activin A was examined in the control plasmid-transfected clones (HS-5S-2, HS-72S-4) and *BCL-2* expressing clones (HS-5B-3, HS-5B-6, HS-72B-16) by MTT assay. As shown in Fig. 2, control clones (HS-5S-2, HS-72S-4) showed cytotoxic response to activin A in a dose-dependent manner. Activin A-sensitivities of control clones were essentially the same as those of parental cell lines (data not shown). *BCL-2* expressing clones (HS-5B-3, HS-5B-6, HS-72B-16) also showed cytotoxic response to activin A, but were less sensitive than the control clones. As demonstrated in Fig. 3, cultivation of HS-5S-2 and HS-72S-4 clones with activin A increased the population of nuclei with reduced DNA content representing apoptotic nuclei. HS-5B-3 and HS-72B-16 clones showed a slight increase in population of apoptotic nuclei. After being cultured without activin A, these clones showed less than 6% in population of nuclei with reduced DNA content. Gel electrophoresis of cellular DNA showed that fragmented DNA was much less in activin A-treated HS-5B-3 and HS-72B-16 clones than in HS-5S-2 and HS-72S-4 clones exposed to activin A (Fig. 4). Thus, expression of *BCL-2* was shown to inhibit activin A-induced apoptosis in mouse B cell hybridoma cells. We tested if activin A-induced apoptosis was mediated by increasing level of BAX. The parental HS-5 and the transfected clones were cultured with activin A for 12 or 24 h and analyzed for *BAX* expression by the immunoblot analysis. As shown in Fig. 5, overexpression of *BCL-2* did not enhance *BAX* expression in HS-5 cell line. Exposure of the parental and transfected HS-5 clones to activin A did not increase BAX levels at least for 24 h, or even slightly decreased its levels.

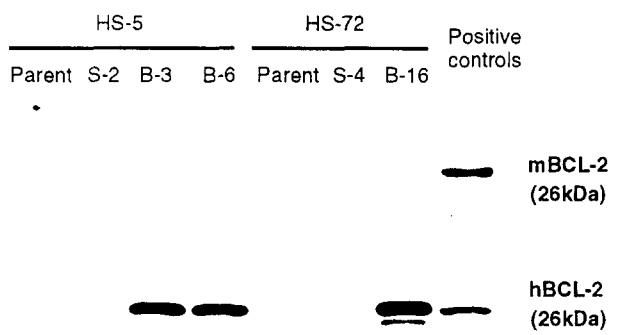

Fig. 1. BCL-2 expression in parental and transfected HS-5 and HS-72 clones. Mouse endogenous BCL-2 (upper panel) and human exogenous BCL-2 (lower panel) expression was studied by immunoblot analysis as described in section 2. M1 cells and U937 cells were used as positive controls to detect mouse BCL-2 and human BCL-2, respectively. mBCL-2, mouse BCL-2; hBCL-2, human BCL-2.

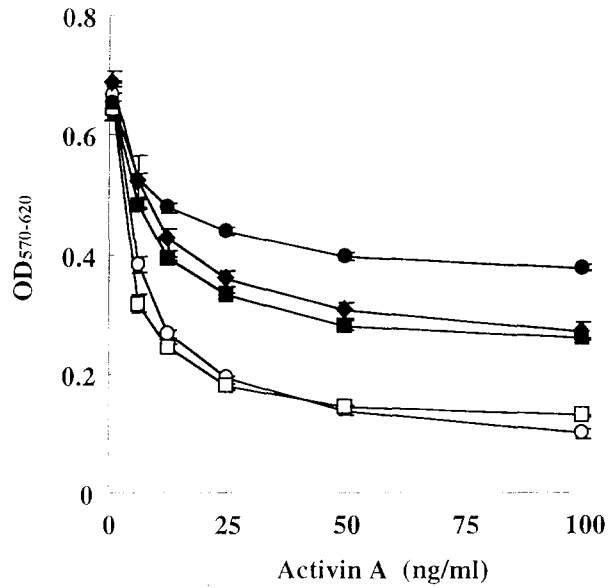

Fig. 2. BCL-2 suppresses activin A-cytotoxicity in hybridoma cell lines. Cells were cultured in the presence of various concentrations of activin A for 48 h and then the MTT assay was performed. □, HS-5S-2; ■, HS-5B-3; ♦, HS-5B-6; ○, HS-72S-4; ●, HS-72B-16.

4. Discussion

We showed that mouse B cell hybridoma cell lines sensitive to activin A-induced cell death expressed undetectable levels of BCL-2 and that overexpression of exogenous BCL-2 suppressed the cell-killing effect of activin A. These results suggested that high sensitivity of hybridoma cell lines to activin A-mediated apoptosis might be attributed to low levels of endogenous BCL-2. Inhibition of activin A-induced apoptosis by BCL-2 was consistent with the idea that most cases of apoptosis, if not all, are mediated by a common or similar process involving members of the *BCL-2* family [13,15]. p53-induced apoptosis was reported to be caused by suppression of *BCL-2* and activation of *BAX* [21,22]. However, activin A did not enhance *BAX* expression in the hybridoma cell lines in the present study. Similar observation was made in M1 mouse myeloblastic cell line stimulated by TGFβ to undergo apoptosis [21]. Other apoptosis-stimulating genes such as *BCL-X*$_S$, *BAD* and *BAK* [15,23–25] might be involved in the induction of apoptosis by the TGFβ superfamily.

Apoptosis is essential in normal development and regulation

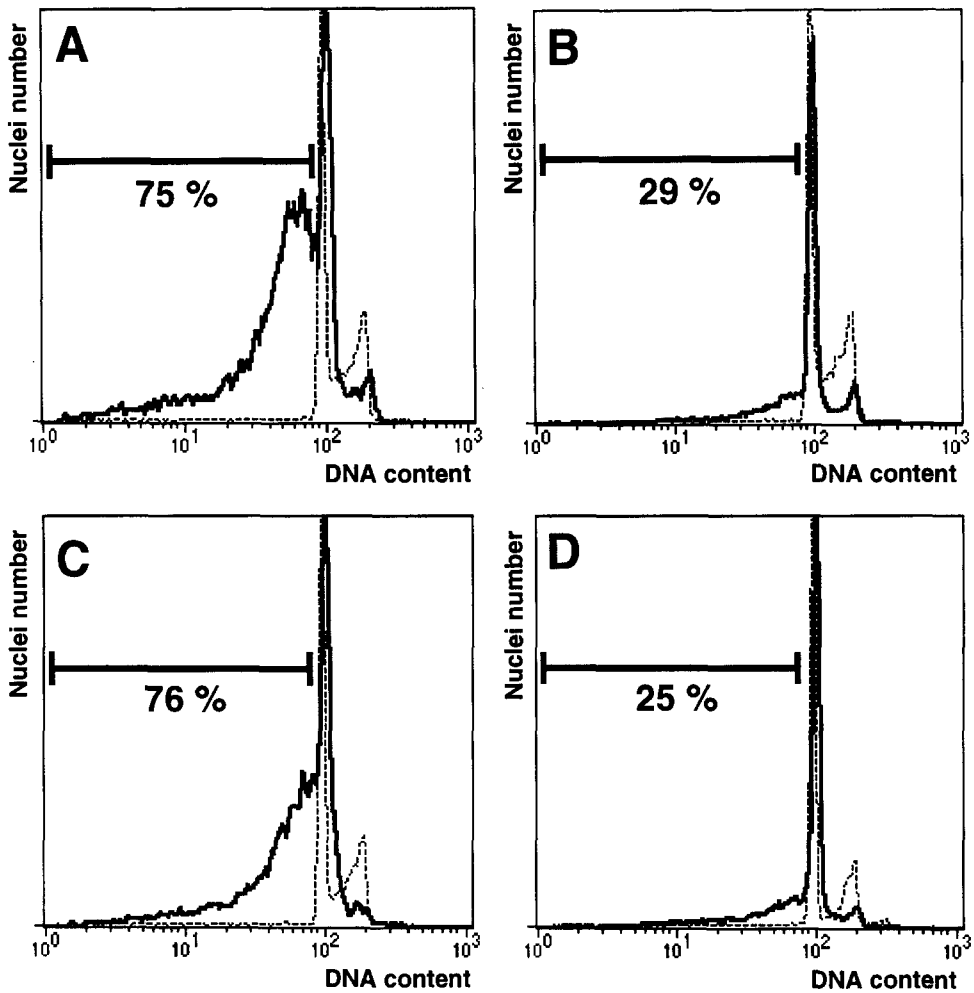

Fig. 3. Analysis of DNA content in transfected hybridoma clones treated with activin A. Transfectants of hybridoma cell lines were cultured with (—) or without (---) activin A (100 ng/ml) for 28 h, stained with propidium iodide and analyzed by flow cytometry. The percentages of hypodiploid nuclei in activn A-treated cells are indicated. Panel A, HS-5S-2; Panel B, HS-5B-3; Panel C, HS-72S-4; Panel D, HS-72B-16.

of the immune system and hematopoiesis [26]. A large amount of information has been accumulated during the last few years contributing to our knowledge of the principles of apoptosis. For the process of elimination of self-reactive B cells, it is likely that induction of tolerance involves apoptosis as a part of a parallel process of negative selection [27]. We reported previously that a factor produced by the mouse macrophage cell line $P388D_1$ cells shows a profound suppressive effect on in vitro proliferation of B lineage cells. In the course of the study, this negative growth effect was at least in part caused by induction of apoptosis. Peptide analysis revealed that the N-terminal amino acid sequence of the factor was identical to that of activin A [10]. Thus, it is likely that activin A may participate in B cell differentiation and immunoregulation through crosstalk between macrophages and B cells at a certain differentiation stage. Further studies are needed to elucidate the precise interaction between B lineage cells and macrophages through activin A-mediated apoptosis.

It is well-known that activins transduce their effects through binding to ActR I and ActR II. Attisane et al. reported that ActR I is transmembrane protein kinases that associate with ActR II to generate derivative heterometric serine/threonine kinase complexes of differential signaling capacities [28]. We showed previously that activin A-induced B cell apoptosis was suppressed by an inhibitor of protein kinases, suggesting that the apoptotic signals from ActR may be mediated by protein kinase and its target protein(s) [10]. Further work is in progress to confirm the identity of the kinase and intracellular signal for B cell death after binding of activin A to the complex of ActR I and ActR II.

Acknowledgements: This work is supported in part by National Institute Post Doctoral Fellowship of the Research Development Corporation of Japan and Grants-in aid for Scientific Research from the Ministry of Education, Science and Culture of Japan.

References

[1] Vale, W., Rivier, J., Vaughan, J., McClintock, R., Corrigan, A., Woo, W., Karr, D. and Spiess, J. (1986) Nature 321, 776–779.
[2] Schubert, D., Kimura, H., LaCorbiere, M., Vaughan, J., Karr, D. and Fischer, W.H. (1990) Nature 344, 868–870.
[3] Eto, Y., Tsuji, T., Takezawa, M., Takano, S., Yokogawa, Y. and Shibai, H. (1987) Biochem. Biophys. Res. Commun. 142, 1095–1103.
[4] Green, J.B.A. and Smith, J.C. (1990) Nature 347, 391–394.

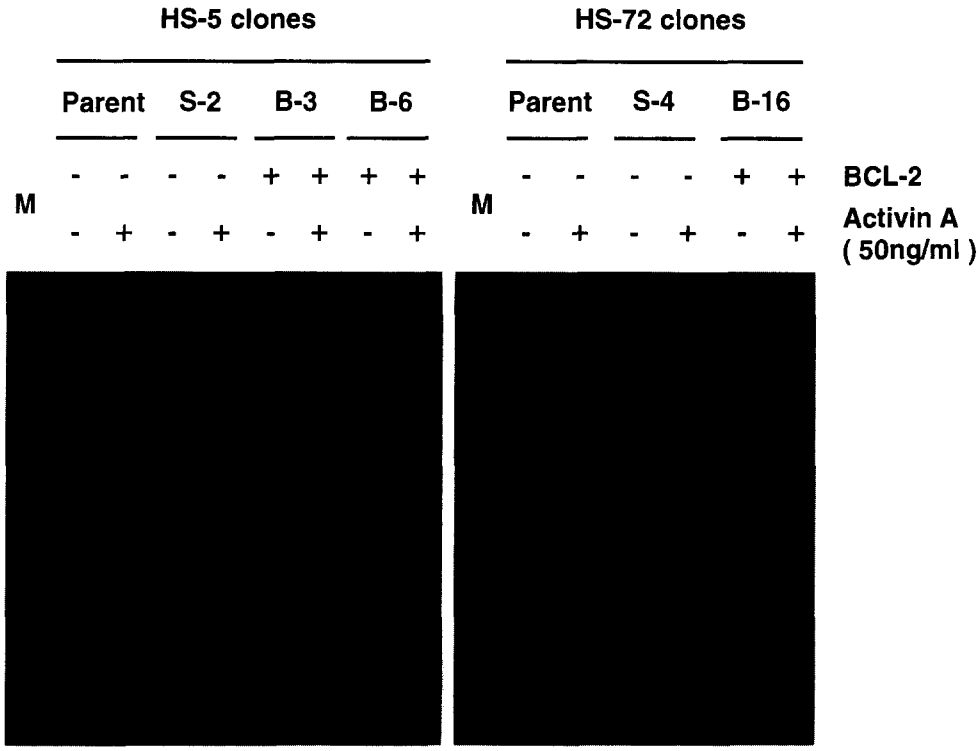

Fig. 4. DNA fragmentation of HS-5 and HS-72 clones treated with activin A. Hybridoma clones (5×10^6) were cultured with or without activin A (50 ng/ml). After being cultured for 24 h, the cellular DNA was isolated as described in section 2. The DNA samples were analyzed by electrophoresis in 2% agarose gels. Lane M, Superladder low dsDNA Marker Kit (Gen Sura Laboratories, Inc., Del Mar, CA).

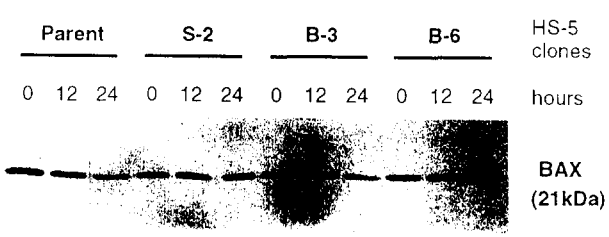

Fig. 5. Activin A does not increase BAX level in B cell hybridoma cells. The cells were cultured with activin A (50 ng/ml) for 12 or 24 h. The parental HS-5, HS-5S-2, HS-5B-3, and HS-5B-6 cells were analyzed for BAX expression by immunoblot analysis.

[5] Thomsen, G., Woolf, T., Whitman, M., Sokol, S., Vaughan, J., Vale, W. and Melton, D.A. (1990) Cell 63, 485–493.
[6] Nishihara, T., Okahashi, N. and Ueda, N. (1993) Biochem. Biophys. Res. Commun. 197, 985–991.
[7] Mathews, L.S. and Vale, W.W. (1991) Cell 65, 973–982.
[8] Attisano, L., Wrana, J.L., Cheifetz, S. and Massagué, J. (1992) Cell 68:, 97–108.
[9] Mathews, L.S. (1994) Endocrine Rev. 15, 310–325.
[10] Nishihara, T., Ohsaki, Y., Ueda, N., Koseki, T. and Etoh, Y. (1995) J. Interferon Cytokine Res. 15, 509–516.
[11] Steller, H. (1995) Science 267, 1445–1448
[12] Tsujimoto, Y. and Croce, C.M. (1986) Proc. Natl. Acad. Sci. USA 83, 5214–5218.
[13] Vaux, D.L., Cory, S. and Adams, J.M. (1988) Nature 335, 440–442.
[14] Gottschalk, A.R., Boise, L.H., Thompson, C.B. and Quintáns, J. (1994) Proc. Natl. Acad. Sci. USA 91, 7350–7354.
[15] Boise, L.H., González-García, M., Postema, C.E., Ding, L., Lindsten, T., Turka, L.A., Mao, X., Nuñez, G. and Thompson, C.B. (1993) Cell 74, 597–608.
[16] Oltvai, Z.N., Milliman, C.L. and Korsmeyer, S.J. (1993) Cell 74, 609–619.
[17] Tsujimoto, Y. (1989) Proc. Natl. Acad. Sci. USA 86, 1958–1962.
[18] Perandones, C.E., Illera, V.A., Peckham, D., Stunz, L.L. and Ashman, R.F. (1993) J. Immunol. 151, 3521–3529.
[19] Moore, K.J. and Matiashewski, G. (1994) J. Immunol. 152, 2930–2937.
[20] Krajewski, S., Krajewski, M., Shabaik, A., Miyashita, T., Wang, H.G. and Reed, J.C. (1994) Am. J. Pathol. 145, 1323–1336.
[21] Selvakumaran, M., Lin, H.-K., Miyashita, T., Wang, H.G., Krajewski, S., Reed, J.C., Hoffman, B. and Liebermann, D. (1994) Oncogene 9, 1791–1798.
[22] Miyashita, T., Krajewski, S., Krajewska, M., Wang, H.G., Lin, H.K., Liebermann, D.A., Hoffman, B. and Reed, J.C. (1994) Oncogene 9, 1799–1805.
[23] Yang, E., Zha, J., Jockel, J., Boise, L.H., Thompson, C.B. and Korsmeyer, S.J. (1995) Cell 80, 285–291.
[24] Chittenden, T., Harrington, E.A., O'Connor, R., Flemington, C., Lutz, R.J., Evan, G.I. and Guild, B.C. (1995) Nature 374, 733–736.
[25] Kiefer, M.C., Brauer, M.J., Powers, V.C., Wu, J.J., Umansky, S.R., Tomei, L.D. and Barr, P.J. (1995) Nature 374, 736–739.
[26] Raff, M.C. (1992) Nature 356, 397–400.
[27] Williams, G.T. (1994) J. Pathol. 173, 1–4
[28] Attisano, L., Cárcamo, J., Ventura, F., Weis, F.M.B., Massagué, J. and Wrana, J.L. (1993) Cell 75, 671–680.