Bcl-2 Inhibits Retinoic Acid–induced Apoptosis during the Neural Differentiation of Embryonal Stem Cells

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Abstract. We report here that all trans-retinoic acid (RA), a classical morphogen, induces apoptosis during the neural differentiation of the embryonic stem cell line P19. The apoptotic cells showed, in addition to DNA cleavage, typical morphological changes including chromatin condensation, nuclear fragmentation, and cytoplasmic vacuolation. These apoptotic changes became obvious by 12 h after the addition of RA. The endogenous expression of bcl-2 in surviving cells was down-regulated during this process, and the compelled expression of bcl-2 by retroviral vectors reduced the number of apoptotic cells. Apoptosis was partially inhibited by adding antisense oligonucleotides against RA receptors (RARs) simultaneously or by transfecting a plasmid vector flanked with a RA-responsive element. Antisense oligonucleotides against retinoid X receptors (RXRs), the receptors for 9 cis-RA, did not inhibit apoptosis induced by all trans-RA. Cycloheximide and actinomycin D, inhibitors of protein and RNA syntheses, respectively, suppressed apoptosis. No changes were seen in the expression of tumor necrosis factors, their receptors, Fas, FasL, p53, or c-myc, molecules which have been suggested to participate in the apoptotic process. Addition of neurotrophins to the culture medium did not affect apoptosis. These findings suggest that the signals mediated by RARs, the differentiation signals themselves, promote expression of molecules essential for apoptosis. Furthermore, we observed that RA induced apoptosis of cerebral neurons from murine embryos in primary culture, which suggests that RA might participate in cell death which occurs during neural development.

Apoptosis is a biological process which causes programmed cell death during development and may participate in various pathological processes. It is characterized by typical morphological changes including chromatin condensation, nuclear fragmentation, cytoplasmic vacuolation, membrane blebbing and cell shrinkage, as well as by the biochemical marker DNA cleavage. Apoptosis eliminates 20–80% of neurons during development (Hamburger and Oppenheim, 1982; Oppenheim, 1991) but leaves neurons which are indispensable for the function of the nervous system. This process seems essential because unnecessary neurons might disturb the development of neural networks. The apoptotic process is also observed under conditions stressful for neurons such as serum/neurotrophic factor deprivation (Greene and Tischer, 1976; Greene, 1978; Batistatou and Greene, 1991) or exposure to neurotoxins (Dispasquale et al., 1991; Edwards et al., 1991) including toxic levels of glutamate (Kure et al., 1991). Apoptosis has been postulated as a model for neuronal death in human neurodegenerative diseases because of morphological similarities at the cellular levels.

Apoptosis is regulated by the balance of various molecules including tumor suppressor genes, protooncogenes, and cell cycle regulators. Among the tumor suppressor genes, Rb and p53 are known to participate in apoptosis. Inhibition of Rb function by gene disruption (Morgenbesser et al., 1994) or by expression of human papilloma virus protein E7 (Pan and Griep, 1994) interferes with terminal differentiation of lens fiber cells, causes apoptosis of those cells, and results in microphthalmia. In this process, Rb acts as an inhibitor of DNA synthesis after withdrawal from the cell cycle and thereby prevent apoptosis. On the other hand, disruption of the p53 gene in mice reduces apoptotic cell death (Clarke et al., 1993; Lowe et al., 1993). Over-expression of p53 causes growth arrest and apoptosis (Yonish-Rouach et al., 1991), and p53 mutations are widely observed in various tumors (reviewed by Culotta and Koslhand, 1993), suggesting that p53 mediates the apoptotic process to suppress the progression of tumors.

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Materials and Methods

Cell Culture and RA Treatment

P19 cells were maintained in α-MEM (Sigma Chemical Co., St. Louis, MO) supplemented with 10% fetal bovine serum (ICN Radiochemicals, Irvine, CA). To induce neural cell differentiation, 1 × 10^6 P19 cells were cultured in 10 cm bacteriological dishes (Nipro) in 10 ml of medium containing 0.1 μM of all-trans-RA (Sigma Chemical Co., St. Louis, MO) for 4 d, transferred to tissue culture dishes (Corning, Corning, NY), and cultured in the absence of RA for 2 d. To induce mesodermal cell differentiation, they were cultured in tissue culture dishes for 2-4 d in the presence of RA.

We used 10⁻⁷ M RA in most experiments because this order of concentration is commonly used to differentiate P19 (Rudnicki and McBurney, 1987) and because it was optimal to observe apoptosis morphologically. However, RA concentrations were changed as described in the text and figure legends when necessary. RA stock solution (10⁻⁴ M all-trans-RA in ethanol) was diluted with the culture medium just before use. Cycloheximide and/or actinomycin-D at concentrations of 1.0 μg/ml and 0.1 μg/ml, respectively, were added for the inhibition of protein/RNA synthesis simultaneously with RA.

Ultrastructural Analysis of Apoptotic Cells

During the procedure for neural differentiation, dying cells were separated from cell aggregates. Culture medium was recovered to a centrifuge tube, and left to stand for 5 min. Cell aggregates dropped to the bottom during this time. The supernatants were carefully removed to new centrifuge tubes, and the floating cells were spun down at 200 g for 10 min. The collected cells were prefixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4), rinsed three times with 0.1 M phosphate buffer, postfixed in 1% OsO₄ in 0.1 M phosphate buffer, and embedded in 1% agarose, dehydrated, and re-embedded in Epon. Semi-thin sections were stained with 0.1% toluidine blue. Ultra-thin sections were contrasted with uranyl acetate and lead citrate.

Estimation of Cell Death

To calculate the percentage cell death, we directly counted the dead cells in preparations stained by trypan blue. P19 cells (1 × 10⁶/bacteriological dish in 10 ml of medium - 10% FBS) or cultured neurons (the culture conditions were described later in Primary Culture of Neurons) were incubated with 10⁻⁷ M of RA for 48 h. Thereafter they were scraped off from the bottom of the dishes and collected with the culture medium. An equal volume of 0.4% trypan blue solution (GIBCO BRL, Gaithersburg, MD) was added to the medium and left for 1 min. The numbers of stained and total cells were then counted within 5 min.

Immunohistochemical Analysis of Differentiated Cells

Immunohistochemistry was performed to determine the characteristics of the differentiated P19 and P19-Bcl-2 stable transformant cells. Antibodies against neuronfilament 160K, GFAP, vimentin, GalC, and fibronecetin were used for this purpose (Neuronal cell typing kit; Boehringer-Mannheim Biochemicals, Indianapolis, IN). These cells were fixed with methanol precooled to −20°C or 5% (vol/vol) acetic acid in ethanol in the case of staining with the anti-GalC antibody.

DNA Preparation and Analysis

Genomic DNA was prepared as described previously (Okazawa et al., 1991). Briefly, dying cells were separated as described above and centrifuged at 200 g for 10 min. The pellets were resuspended in a 10-fold volume of RSB solution (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 1.5 mM MgCl₂), left on ice for 10 min, homogenized in RSB with 0.5% NP-40, centrifuged at 800 g for 5 min. The resulting nuclear pellets were suspended in an equal volume of extraction buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 100 mM EDTA) containing SDS (final 0.5%) and protease K (final 200 ng/ml), incubated at 37°C for 6 h, extracted with phenol/chloroform (1:1) for more than 12 h, and centrifuged. The aqueous phase was recovered, and dialyzed with TE (10 mM Tris-Cl, pH 7.5, 1 mM NaCl/EDTA) for 1 d. The concentration of recovered DNA samples were checked, and 1-μl aliquots of each DNA sample were separated on 3% nuciege/agarose (3:1) gels.

DNA Fragmentation Assay

This assay was performed according to the reported method (Sellins and Cohen, 1987). 1 × 10⁶ cells were harvested by centrifugation at 200 g for 10 min. The pellet was lysed with 0.4 ml hypotonic lysis buffer (10 mM Tris, 1 mM EDTA, pH 7.5) containing 0.2% Triton-X 100, and lysates
were centrifuged at 13,000 g for separate intact from fragmented chromatin. The supernatant, containing fragmented DNA, was placed in a separate tube, and both pellets were resuspended in 10 ml glacial acetic acid; Sigma Chemical Co.) was added to each tube. After overnight color development, optical densities of the samples were read at 600 nm. For the analysis of embryonic nuclei in primary culture, 1 x 10^5 cells were used for the reaction scaled down to 1/10x vol.

**RNA Preparation and Northern Blotting Analysis**

RNA was prepared similarly to the step for separating nuclei. After centrifugation, Tris-HCl, pH 9.0 (final 100 mM), SDS (final 0.5%), and NaCl (final 100 mM), were added to the supernatant followed by extraction with phenol/chloroform, and precipitation with ethanol. Poly-A^+ RNA was separated by oligo-dT sepharose column chromatography (Pharmacia Fine Chemicals, Piscataway, NJ) according to the supplier's protocol. 20-μg aliquots of each RNA sample were separated on 1% agarose gels in lx MOPS buffer (20 mM 3-[N-morpholino] propane-sulfonic acid, 5 mM sodium acetate, pH 7.0, 1 mM Na2EDTA) containing 4% formaldehyde, and blotted onto Hybond N (Amersham Corp., Arlington Heights, IL) with a vacuum blotter (Pharmacia Fine Chemicals). The filters were hybridized with a DNA fragment of total RNA, 0.1 μM of both directional primers, 1× RT-PCR buffer consisting of 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl2, 0.1 mg/ml gelatin, 0.1% Triton X-100, 0.25 mM dNTPs, 5 U RNase inhibitor (Boehringer-Mannheim Biochemicals), 2 U AMV-reverse transcriptase XL (Life Science Inc., Gaithersburg, MD), and 2 U Taq polymerase (Takara), were mixed in a total volume of 25 μl. The solution was incubated in a 37°C water bath for 1 h. The products of the amplification were digested with restriction enzymes and analyzed on 1% agarose gels. The amplified products were digested and subcloned into M13mp18 or pBluescriptSK +, which were radiolabeled with a random primer labeling kit (Boehringer-Mannheim). The amplified products were digested and subcloned into pBluescriptSK +. The primers used were 5'-AAGAATTCGG-CAGAACAGGGTATG (from 1832 to 1848 of the mouse bcl-2 gene, Negrini et al., 1987), 5'-AAGATCGGAGAGACCAGAGAG (from position 2137 to 2119). The expected size of the amplified product was 304 bp.

**Inhibition of Apoptosis by Antisense Oligonucleotides**

Antisense and sense oligonucleotides were synthesized by using an Applied Biosystems (Foster City, CA) oligonucleotide synthesizer, and purified by gel filtration. The sequences of antisense oligonucleotides were as follows, RARα1: 5'-CTCCACAGCTGTAC, RARβ1: 5'-GGTGCCTCGTGGTCACT, RARβ2: 5'-ATTCCAGACGTAAC, RARγ: 5'-CTTCTATTGGGCGCAT, retinoid X receptor (RXR)x: 5'-GAAATGTTTGGTGTCCAT, RXRα: 5'-GGAATAATFFCCATAC, RXRβ1: 5'-GTGGCTGCTGGTGCTCAT, RXRγ: 5'-GGTGCCGGGGTGGCAGATT, RXRγ: 5'-GGAATATTCTTAC-ACAT, etc. These were designed based on the previous reports (Zelen et al., 1989; 1991; Leroy et al., 1991; Mangelsdorf et al., 1992) to overlap the initiation codons of receptors. Sense oligonucleotides were made in the reverse orientations. Each oligonucleotide was added at 10 μM to the RA-containing culture medium and exchanged every 24 h. The optimal concentration was determined by preliminary experiments using various amounts of the oligonucleotides: 0.1, 1, 5, 10, and 20 μM. The effect on apoptosis reached a plateau at 20 μM. Stability of the oligonucleotide was checked before use by 0.1 μM of nucleotide. The oligonucleotide was labeled with 32P-y-ATP and polyadenylate kinase (Toyobo, Tokyo, Japan) was incubated in α-MEM medium with 10% FBS at 37°C for 24 h. Degradation was estimated by polyacrylamide gel electrophoresis as described (Akkat et al., 1991). Percentage of the intact oligonucleotide band was calculated. The mean ± SD made in 4 independent experiments was 32 ± 2% in RARα1 sense, 31 ± 2% in RARα1 antisense, 33 ± 2% in RARα2 sense, 34 ± 2% in RARα2 antisense, 32 ± 3% in RARβ1 sense, 30 ± 3% in RARβ1 antisense, 34 ± 2% in RARβ2 sense, 30 ± 2% in RARβ2 antisense, 30 ± 2% in RARγ sense, 31 ± 2% in RARγ antisense, 32 ± 2% in RARγ sense, 33 ± 2% in RXRα sense, 32 ± 2% in RXRα antisense, 33 ± 2% in RXRβ sense, 32 ± 2% in RXRβ antisense, 30 ± 2% in RXRγ sense, and 34 ± 2% in RXRγ antisense. The stability was not statistically different among these oligonucleotides.

**Immunoblot Analysis**

For immunoblot analysis, 4815-selected cells were isolated, amplified to confluence in 10 cm tissue dishes, and scraped into PBS (pH 7.4). The cells were collected by centrifugation at 200 g for 10 min, and the resulting pellets were suspended in ice-cold lysis buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100) containing proteinase inhibitors. After incubation on ice for 10 min, samples were centrifuged at 15,000 g for 20 min. Aliquots of Triton-soluble fractions (each 100 μl) were subjected to SDS-PAGE, blotted onto Hybond ECL (Amersham Corp.), and incubated with anti-human Bcl-2 monoclonal antibody (Santa Cruz), and then detected using an ECL-kit (Amersham Corp.).
Figure 1. (a) DNA cleavage was induced by RA during the neural differentiation of P19 cells by RA. Genomic DNAs of dying cells were analyzed on 3% nusieve-agarose gels 48 h after RA treatment. P19 cells, cultured in bacteriological dishes in the presence of RA at various concentrations, form aggregates which differentiate into neural cells after being transferred to tissue dishes (Rudnicki and McBurney, 1987). During this process, cells which lose their viability detach from the cell aggregates. These dying cells were separated, and their genomic DNAs were analyzed. DNA cleavage was induced by treatment with 10^{-9}-10^{-5} M RA (lanes 4-8). Only a smear of RNA was observed at RA concentrations below 10^{-9} M (lanes 1-3). (b) Observation of DNA cleavage with time. 10^{-7} M RA was added to the culture medium and partial DNA cleavage was detected after 6 h (lane 1). The typical DNA ladder formation at 180 bp was firstly observed 12 h after the addition of RA (lane 2). DNA cleavage was attenuated after P19 cells were transferred to tissue culture dishes and they ceased division and differentiated into neurons (lanes 6 and 7). The medium was changed at each analysis to collect the cells that had newly lost viability between time points. (c) DNA fragmentation assay showing the rapid increase of DNA leakage from nuclei. When P19 cells were treated with 10^{-9} or 10^{-7} M RA but not with 10^{-11} M RA, the percentage of DNA outside of nuclei, which was calculated as described in Materials and Methods, increased promptly.

**Primary Culture of Neurons**

At first, the meninges were removed from the brain surface of E16 murine embryos. Small pieces of cerebral cortex (3 mm x 3 mm x 1 mm depth) were dissected from the cerebrum, incubated in 0.25% trypsin (Immuno-Biological Laboratories) at 37°C for 5 min, and dissociated by gentle pipetting in Hank's Ca^{2+}, Mg^{2+}-free BSS (Sigma Chemical Co.) containing 0.05% DNase I (Sigma Chemical Co.). Samples were then rinsed and re-suspended in α-medium (Sigma Chemical Co.) supplemented with 10% fetal bovine serum (ICN Radiochemicals) and 5% donor horse serum (CELLect; ICN Radiochemicals), and then seeded in poly-L-lysine hydrobromide (Sigma Chemical Co.)-coated dishes. The same lots of serum were used for analysis. For the estimation of neuronal survival, we seeded 1,000 neurons per single well in 16-chambers tissue slides (Corning). RA or oligonucleotides, at concentrations described in the figure legends, were added to the medium as appropriate.

**Nick End-Labeling Method**

Nick end-labeling method was performed with a MEBSTAIN Apoptosis kit (MBL) basically according to the commercial protocol. Cultured neurons were fixed by incubation with precooled methanol at −20°C for 10 min, washed with PBS three times, and incubated in 40 μg/ml proteinase for 10 min at 37°C. After washing with TdT buffer, nicked DNA ends were labeled with biotin-dUTP by terminal deoxynucleotide transferase. The enzymes and solutions used above were contained in the kit. The tissue slides were washed with water twice, incubated with avidin-peroxidase (Sigma Chemical Co.), washed with PBS twice, and stained with DAB (3, 3′-diaminobenzidine; Sigma Chemical Co.).

**Statistics**

InStat 2.0 computer software (Macintosh) was used for statistical analyses.

**Results**

**RA Induces Apoptosis during the Neural Differentiation of P19 Cells**

P19 is one of the most commonly used embryonic cell lines for the analysis of differentiation as it possesses the characteristics of pluripotent stem cells (Jones-Villeneuve et al., 1983; Rudnicki and McBurney, 1987), and differentiates into neurons and glial cells, when treated with RA in the presence of cell-to-cell contact (Jones-Villeneuve et al., 1983; Rudnicki and McBurney, 1987). The differentiated P19 cells express various neuronal markers like GABA, GABA-transaminase, glutamic acid decarboxylase, neuropeptide Y, serotonin, DOPA decarboxylase, somatostatin, and ionotropic glutamate receptor (Ray and Gottlieb, 1994; Staines et al., 1994). RARs and RXRs have been proposed to mediate RA-induced signals based on their abundant expression (Pratt et al., 1990; Bain et al., 1994). We have noticed in previous experiments (Okamoto et al., 1990; Okazawa et al., 1991) that a large number of cells tend to die during the neural differentiation of P19 cells by RA, and we suspected this phenomenon may be apoptosis.

Generally, multiple pair-wise comparisons were performed using analysis of variance and Bonferroni t test among groups of data.
Figure 2. Ultrastructure of dying P19 cells indicating apoptosis. P19 cells which lost their viability during RA-induced neural differentiation were collected and observed by electronmicroscopy. Various phases of apoptosis were found in different cells (a). At higher magnifications, chromatin condensation (b), cytoplasmic vacuolation, disruption of the nuclear membrane, nuclear fragmentation (c), and rupture of the cytoplasmic membrane (d) were observed. $10^{-9}$ M RA was used for the morphological analysis presented here; however, we observed similar apoptotic changes on treatment with $10^{-9} - 10^{-5}$ M RA. The percentage of apoptotic cells changed according to the concentration of RA used.

Firstly, to test this impression, genomic DNA of the dying cells was analyzed on agarose gels on the second day of RA treatment. As expected, DNA fragmentation at about 180 bp was observed on treatment with RA from $10^{-9} - 10^{-5}$ M (Fig. 1 a). DNA fragmentation was confirmed by 12 h after exposure to $10^{-7}$ M RA (Fig. 1 b) and was attenuated after day 5 when P19 cells ceased cell division and differentiated into neurons. This observation was quantitatively confirmed by DNA fragmentation assay (Burton, 1956; Sellins and Cohen, 1987). This assay detected the cellular change earlier than the agarose gel electrophoresis, because it observed the DNA leakage from nuclei (Fig. 1 c).

Secondly, the ultrastructural appearance of the dying cells was evaluated. Almost all the dying cells displayed typical apoptotic changes including chromatin condensation, nuclear fragmentation, and cytoplasmic vacuolation when treated with $10^{-7}$ M RA (Fig. 2). The progression of apoptosis from the chromatin condensation to the rupture of the cytoplasmic membrane could be followed in different cells (Fig. 2). A subpopulation of cells showed degenerative changes when treated with a higher concentration of RA (data not shown).

These morphological changes and the DNA fragmentation were similarly induced by another form of RA treatment that evoked differentiation of P19 cells predominantly into endodermal cells. With this treatment, simply culturing P19 cells in tissue dishes for 2 d, apoptosis was observed within 12 h with RA at concentrations above $10^{-10}$ M (data not shown). This observation suggests that RA-induced apoptosis is not restricted to a specific orientation of differentiation.

Apoptotic Signaling Pathway of RA

All trans-RA binds to RARs, while 9 cis-RA binds to RXRs and RARs (for review see Chambon, 1994). Both of these receptor families possess three members, $\alpha$, $\beta$, and $\gamma$, which act as transcription factors when activated by their ligands (for review see Mangelsdolf, 1994). Therefore, it is possible that the RA-induced signal for the apoptosis is similarly transduced to the nucleus by these receptors and thereby elicits expression of genes which cause apoptosis.

To determine whether RARs mediate the apoptotic sig-
nal, we performed three sets of experiments. Firstly, we synthesized antisense oligonucleotides against RARs (as well as RXRs) and observed whether they inhibited apoptosis. Oligonucleotides against specific members of the RAR family or against specific transcripts of an RAR family member reduced apoptotic cells mildly, while they obviously suppressed apoptosis with a statistical significance when used together (Fig. 3). As all the members of RAR and RXR families are expressed during the differentiation of P19 cells evoked by RA (Jonk et al., 1992; Bain and Gottlieb, 1994), these findings suggest that multiple members of the RAR family, but not of the RXR family for 9 cis-RA, participate in the apoptotic signal transduction from all trans-RA.

Secondly, we transfected P19 cells with a CAT reporter plasmid containing RA response elements (pRARE-}

They were cultured in the presence of 10^{-7} M of RA for additional 24 h and trypan blue-positive cells were counted microscopically. CAT activities were assayed simultaneously using another dish of P19 cells treated similarly. The percentage cell death was determined by the same formula as that shown in Fig. 3. The relative CAT activity was a ratio to the mean value of the transfections using 5 μg of plasmid. The relationship between the percentage cell death and the relative CAT activity is shown in the figure. The negative relationship between these two parameters suggests that the RA response element absorbs the apoptotic signals mediated by some retinoic acid receptors.
Expression of *bcl-2* was not detected by Northern blot analysis during RA-induced neural differentiation of P19 cells. PolyA⁺ RNA was prepared from differentiated P19 cells cultured in the presence of RA and undifferentiated P19 cells cultured in the absence of RA. Expression of the dopamine receptor D₁A gene was obviously up-regulated in differentiated P19 cells, while expression of *bcl-2* was not detected even when the x-ray film was exposed for 7 d.

(b) Expression of *bcl-2* was detected by RT-PCR but suppressed further by RA. P19 cells were cultured in bacteriological dishes in the presence or absence of 10⁻⁷ M RA for 12 or 24 h. 1 µg of total RNA was used for each RT-PCR. G3PDH was amplified as a reference (lanes 1, 2, 5, and 6). *bcl-2* (lane 4 or 8) was down-regulated obviously when compared to its reference (lane 3 or 7) and G3PDH (lane 2 or 6) amplified from the same RNA samples. The genes corresponding to these bands were confirmed by sequencing analysis.

(c) Quantitative analysis of *bcl-2* expression. *bcl-2* and G3PDH bands were quantified using an image analyzer. Percentage *bcl-2* amount was calculated at each time point by the formula $(\text{bcl-2 amount in the presence of RA} \times \text{G3PDH amount in the absence of RA})/(\text{bcl-2 amount in the absence of RA} \times \text{G3PDH amount in the presence of RA})$. Values represent the mean ± SD determined in triplicate experiments. *P < 0.01 compared to the value calculated with *bcl-2* before treatment.

(d) Expression of the genes related to *bcl-2* did not change during RA-induced neural differentiation. RNA samples were prepared from non-differentiated P19 cells or 24 h treatment with 10⁻⁷ M RA and aggregation. *bax*, *bad*, and *bag-1* gene expression was analyzed by RT-PCR. Specific bands for *bad* and *bag-1* stayed constant (arrowheads). *bax* gene was not amplified in both conditions, while a nonspecific band was observed (dot).

Figure 6. (a) Expression of *bcl-2* was not detected by Northern blot analysis during RA-induced neural differentiation of P19 cells. PolyA⁺ RNA was prepared from differentiated P19 cells cultured in the presence of RA and undifferentiated P19 cells cultured in the absence of RA. Expression of the dopamine receptor D₁A gene was obviously up-regulated in differentiated P19 cells, while expression of *bcl-2* was not detected even when the x-ray film was exposed for 7 d. (b) Expression of *bcl-2* was detected by RT-PCR but suppressed further by RA. P19 cells were cultured in bacteriological dishes in the presence or absence of 10⁻⁷ M RA for 12 or 24 h. 1 µg of total RNA was used for each RT-PCR. G3PDH was amplified as a reference (lanes 1, 2, 5, and 6). *bcl-2* (lane 4 or 8) was down-regulated obviously when compared to its reference (lane 3 or 7) and G3PDH (lane 2 or 6) amplified from the same RNA samples. The genes corresponding to these bands were confirmed by sequencing analysis. (c) Quantitative analysis of *bcl-2* expression. *bcl-2* and G3PDH bands were quantified using an image analyzer. Percentage *bcl-2* amount was calculated at each time point by the formula $(\text{bcl-2 amount in the presence of RA} \times \text{G3PDH amount in the absence of RA})/(\text{bcl-2 amount in the absence of RA} \times \text{G3PDH amount in the presence of RA})$. Values represent the mean ± SD determined in triplicate experiments. *P < 0.01 compared to the value calculated with *bcl-2* before treatment. (d) Expression of the genes related to *bcl-2* did not change during RA-induced neural differentiation. RNA samples were prepared from non-differentiated P19 cells or 24 h treatment with 10⁻⁷ M RA and aggregation. *bax*, *bad*, and *bag-1* gene expression was analyzed by RT-PCR. Specific bands for *bad* and *bag-1* stayed constant (arrowheads). *bax* gene was not amplified in both conditions, while a nonspecific band was observed (dot).

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CAT) and observed its effect on apoptosis. We assumed that the transfected RA response element should compete for binding to RARs, with endogenous RA response elements which regulate the genes inducing apoptosis, and should thereby inhibit apoptosis. Since this experiment was dependent on the transfection efficiency, we used the CAT activity as a marker for the efficiency and corrected the effect of pRARE-CAT on the RA-induced apoptosis. As expected, we observed an inverse correlation between the percentage cell death and the CAT activity (Fig. 4). This result is consistent with the idea that RARs mediate the apoptotic signal of RA. Although the effect was less prominent as that of antisense oligonucleotides, this can be explained by the difference in transfection efficiency between the two methods.

Finally, we observed the effects of protein/RNA synthesis inhibitors on RA-induced apoptosis. We added cycloheximide for the inhibition of protein synthesis and actinomycin-D for the inhibition of RNA synthesis to the culture media. Numbers of apoptotic cells were obviously de-
Increased by both treatments (Fig. 5). These observations suggest that RA-induced apoptosis requires de novo synthesis of causative gene products through transcriptional activation by RARs.

**bcl-2 Is Repressed Strictly during RA-induced Differentiation/Apoptosis**

*bcl-2* expression was observed during the RA-induced differentiation for the following two reasons. Firstly, the importance of Bcl-2 function in neuronal death has been demonstrated by recent studies. Bcl-2 rescues neurons from the programmed cell death during development (Garcia et al., 1992), and inhibits neuronal apoptosis caused by neurotrophic factor deprivation (Allsopp et al., 1993), serum deprivation (Mah et al., 1993), or exposure to multiple neurotoxic chemicals including glutamate (Zhong et al., 1993; Reed, 1994). Secondly, the expression of *bcl-2* in neurons was confirmed at the cellular level (Castren et al., 1994; Merry et al., 1994). As *bcl-2* expression is regulated developmentally (Merry et al., 1994; Castren et al., 1994; Ferrer et al., 1994), this discrepancy could have been due to the difference in maturity between differentiated P19 cells and neurons in vivo.

**Dysregulated *bcl-2* Expression Inhibits Apoptosis Induced by RA**

To test whether dysregulated *bcl-2* expression can rescue P19 cells from RA-induced apoptosis, we established stable transformants of P19 cells which express *bcl-2* constitutively. P19 cells were transfected by a retroviral vector and selected with G418. The resultant clones (P19bc12-7, -8, and -16) were confirmed to express the Bcl-2 protein in both undifferentiated and differentiated states by Western blot analysis (Fig. 7a) and to possess a similar differentiation potential to P19 cells by morphological and immunohistochemical analyses (data not shown). We next observed the effects of dysregulated expression of *bcl-2* on RA-induced apoptosis using these clones. The percentage cell death, which was estimated 48 h after addition of RA, was clearly repressed in these clones when compared to the parental cell line (Fig. 7b). This suggested that *bcl-2* expression can partially inhibit RA-induced apoptosis.

We addressed whether P19bc12s and P19 cells could equally differentiate into neurons. When exposed to $10^{-7}$ RA in tissue culture dishes, P19bc12s cells develop a small number of neurons on the layer of nonneuronal cells (Fig. 8a). When aggregated and exposed to $10^{-7}$ RA in bacteriological dishes, they develop numerous neurons in subsequent culture using tissue culture dishes (Fig. 8b). These features in differentiation were similar to those of P19 cells. The mean percentages of differentiated neurons were almost equal among the cell lines: 37% in P19, 36% in P19bc12-7, 38% in P19bc12-8, and 34% in P19bc12-16, suggesting that the overexpression of *bcl-2* did not directly influence on the differentiation potential of P19 cells. As a result, the number of differentiated neurons from P19bc12s increased correspondingly to the reduction of apoptosis (Fig. 8c).
Figure 8. Neural differentiation of P19 cells constitutively expressing bcl-2. 1 x 10^5 P19bcl2-7 cells were cultured with 10^{-7} M RA in a 10 cm tissue grade culture dish for 4 d (a) or cultured with 10^{-7} M RA in a 10 cm bacteriological grade dish for 2 d and additionally in a 10 cm tissue grade dish for 2 d in the absence of RA (b). Cells were incubated with a monoclonal anti-neurofilament 160K antibody (Boehringer-Mannheim) and detected by a biotinilated secondary antibody and 3,3'-diaminobenzidine. In the former culture (a), small numbers of neurons extending neurites (arrowheads) were observed on the non-neuronal cell layer. Large numbers of neurons were differentiated in the latter culture (b). (c) Numbers of differentiated neurons increased in P19bcl2s. P19 and P19bcl2s were differentiated by RA/aggregation treatment as described in b. On day 4, the differentiated neurons were counted after immunostaining and the mean percentage of neurons was determined in eight fields. Relative neuronal number was calculated by the formula: (total cell number of each P19bcl2 in a dish \times mean percentage of neurons in each P19bcl2)/(total cell number of P19 in a dish \times mean percentage of neurons in P19). Values represent the mean ± SD made in four independent cultures.

Apoptosis Is Not Associated with Changes in Cytokine Expression

It is possible to speculate that RA causes apoptosis either by repressing signals of neurotrophins or by enhancing apoptotic signals of some cytokines. With regard to neurotrophic signals, the expression of trkB, the receptor for BDNF and NT-4/5, was shown to be increased during the neural differentiation of P19 cells by RT-PCR (Salvatore et al.).
et al., 1995; and our unpublished observation). Therefore, supplementing the culture medium with neurotrophins should inhibit apoptosis, if neurotrophins play an important role in RA-induced apoptosis. However, this possibility was excluded because addition of NGF, BDNF, NT-3, or NT-4 to the culture medium and the simultaneous use of all these neurotrophins had no effect on apoptosis (data not shown). We next analyzed the expression of molecules that have been suggested to participate in cell death. RT-PCR analysis indicated that p53, c-myc, TNF-receptor-I, and FasL expression were stable during RA-induced neural differentiation. TNF-α, TNF-β, TNF-receptor II, and Fas expression were hardly detected by this analysis (data not shown). These findings do not support the idea that apoptosis is a secondary phenomenon caused by a change in cytokine systems.
TNF-receptors I and II were reportedly up-regulated by RA in a neuroblastoma cell line (Chambaut-Guerin et al., 1995). However, their data showed the down-regulation of TNF-receptor I from days 4 to 6 and again the up-regulation from days 8 to 14, suggesting unstable expression of this receptor. The up-regulation of TNF-receptor II mRNA was observed only transiently on day 1. In our system using P19, the changes of TNF-receptors were not observed statistically. This discrepancy between the cell lines might reflect the difference in cellular maturity.

**RA Induces Apoptosis of Embryonic Neurons in Primary Culture**

To test whether RA induces neuronal apoptosis in vivo, neurons were dissociated from the murine cerebrum at embryonic day 16 (E16). Survival of these neurons in primary culture was estimated 48 h after dissociation, by direct cell counting under phase-contrast microscopy, by immunostaining with anti-neurofilament 160K antibody, and by nick end labeling (NEL).

The survival of neurons from the cerebrum of E16 embryos was decreased obviously by RA treatment (Fig. 9). This neuronal death was judged to be apoptosis from the obvious nuclear staining shown by NEL experiments (Fig. 9) and the DNA fragmentation detected by agarose gel electrophoresis (Fig. 10 a). The qualitative DNA fragmentation assay confirmed that apoptosis occurred when exposed to 10⁻⁹ or 10⁻⁷ M RA (Fig. 10 b). A slight increase in the percentage DNA fragmentation of the reference group was observed due to the inevitable death of primary neurons in culture (Fig. 10 b). This finding corresponded to the observations that a small portion of neurons morphologically died in the absence of RA (Fig. 9) and that the DNA of nontreated neurons showed a faint smear in agarose gel electrophoresis (Fig. 10 a). However, it was concluded undoubtedly that RA promoted apoptosis judging from the DNA ladder formation in agarose gel electrophoresis and the substantial increase of apoptosis observed by DNA fragmentation assay (Fig. 10 b).

Neurons in primary culture and P19 cells were almost similar in the range of effective concentration of RA to induce apoptosis. RA-induced apoptosis of embryonic neurons in culture was similarly inhibited by the use of antisense oligonucleotides against RARs (Fig. 11). Antisense oligonucleotides against RXRs were not capable to inhibit apoptosis in both cases. These findings suggest that P19 cells and embryonic neurons used common signals in the RA-induced apoptosis.

**Discussion**

RA is one of the classical morphogens which induce cellular differentiation and participate in morphological pat-
tern formation. From the analysis of limb formation, RA is suspected to modulate or collaborate with the other morphogenetic signals such as the sonic hedgehog gene (Shh) (Echelard et al., 1993; Riddle et al., 1993), homeobox genes (Morgan et al., 1992; Dolle et al., 1993; Charite et al., 1994), fibroblast growth factor 4 (FGF4) (Niswander et al., 1992, 1994), and bone morphogene proteins (BMPs) (Niswander et al., 1993; Francis et al., 1994) to function as a morphogen (reviewed by Maden 1994; Tabin, 1995). It is well known that apoptosis is induced at the inter-digit areas during this limb formation. However, how RA contributes to this process has not been determined.

RA induces differentiation of various embryonic stem cell lines. P19 cells are differentiated by RA into neuronal cells which express various neuron-specific molecules and display typical morphological characteristics (Rudnicki and McBurney et al., 1986). We found that apoptosis occurs during this neural differentiation. Our experiments indicated that apoptosis was induced at least by 10^{-9} M RA. It corresponds well to the minimum concentration of RA which efficiently activates RARs-mediated gene transcription (Zelent et al., 1989; Giguere et al., 1990). Furthermore, our approaches inhibiting the RARs function succeeded in partially suppressing apoptosis. These findings suggest that RARs-mediated signal transduction play a major role in this apoptosis. RARs should regulate expression of the genes affecting apoptosis as inhibitors of RNA/protein synthesis repressed the RA-induced apoptosis.

Among the various molecules affecting apoptosis, expression of bcl-2 was selectively suppressed during RA-induced apoptosis of P19 cells. In addition, dysregulated expression of bcl-2 by a retroviral vector inhibited apoptosis. However, the alteration of bcl-2 expression would not be a direct cause of apoptosis because undifferentiated P19 cells which show low levels of bcl-2 expression possess high viability. This suggests that the down-regulation of bcl-2 provides conditions where the RA-induced apoptosis can progress without difficulty. Further investigations are necessary to elucidate the mechanisms which positively regulate RA-induced apoptosis.

RA-induced apoptosis of P19 cells possesses several unique characteristics: firstly, both neural differentiation and apoptosis progress simultaneously; secondly, apoptosis is induced by a morphogen which possibly acts in vivo; and thirdly, this apoptosis is Bcl-2-repressible like the programmed cell death of neurons in vivo (Garcia et al., 1992). In these features, our system mimicked the apoptosis which occurs during neural differentiation. This similarity was further confirmed because RA actually induces apoptosis of embryonic neurons. Meanwhile, it should be noted that the RA-induced apoptosis of P19 cells differs from the programmed neuronal death seen during development; the apoptotic process begins immediately after the RA treatment when P19 cells have not yet displayed neuronal characteristics, and is attenuated after the differentiated P19 cells cease cell division. This is in contrast to the programmed neuronal death which occurs just after the arrival of their axons in the target field and therefore after partial maturation of neurons (reviewed by Barde, 1989).

RA-induced apoptosis has been observed in other types of cells recently, and was first reported in HL-60 granulocytes (Park et al., 1994). Interestingly, bcl-2 is repressed during differentiation of HL-60 by RA. Furthermore, dysregulated expression of bcl-2 inhibits apoptosis similarly in HL-60 (Park et al., 1994). The second case has been reported in another embryonic carcinoma cell line, F9, during differentiation to visceral endodermal cells (Atencia et al., 1994). These observations suggest that RA could act as a ubiquitous signal triggering both apoptosis and differentiation in various tissues.

The natures of the triggers of the programmed cell death which occurs in vivo during neural development are not known. We suppose that RA (or other retinoids) might act as an endogenous apoptotic signal during the development of the nervous system based on the following observations. Firstly, RA has been demonstrated to be present in the floor plate of the neural tube of embryos where apoptosis occurs during development (Hogan et al., 1992). Secondly, the receptors for RAs, RARs and RXRs, as well as cellular retinol binding proteins (CRBPs) are expressed widely throughout the nervous system during development (Maden et al., 1990; Ruberte et al., 1992, 1993; Dolle et al., 1994). Thirdly, the ability of low levels of RA to trigger apoptosis during the neural development of stem cells was demonstrated in the present study.

We also expect that our system will be useful for the identification of specific molecules that are indispensable for the apoptosis induced by steroid receptors. It is well known that glucocorticoids induce Bcl-2-repressible apoptosis (Sentman et al., 1991; Strasser et al., 1991; Miyashita and Reed, 1992; Siegel et al., 1992). Androgen receptor, when its CAG repeat is expanded, causes death of motor neurons leading to a motor neuron disease in humans (LaSpada et al., 1991). These observations regarding steroid receptors and our present findings concerning RARs suggest that these receptors might share a common pathway of cell death especially in neurons. Analysis of the downstream signal transduction of the RA-induced apoptosis and comparison between these different systems might be useful for understanding the general mechanism of neuronal death.

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