Bcl-2 accelerates retinoic acid-induced growth arrest and recovery in human gastric cancer cells

Hua-Kang CHOU*†‡, Show-Li CHEN*, Chung-Te HSU§, You-Chen CHAO‡ and Yeou-Ping TSAO*¶¹

*Department of Microbiology and Immunology, National Defense Medical Center, Taipei, Taiwan, Republic of China, †Graduate Institute of Medical Science, National Defense Medical Center, Taipei, Taiwan, Republic of China, ‡Division of Gastroenterology, Tri-Service General Hospital, National Defense Medical Center, Taipei, Taiwan, Republic of China, §Division of Gastroenterology, Poh Ai Hospital, I-Lan, Taiwan, Republic of China, and ¶Department of Ophthalmology, Chang Gung Memorial Hospital and Chung Gung University, Taoyuan, Taiwan, Republic of China

The role of Bcl-2 as an anti-apoptotic protein has been well documented. In the present work, we present evidence that Bcl-2 may also be involved in cell growth regulation. SC-M1 is a unique cell line which responds to retinoic acid (RA) treatment with reversible growth arrest [Shyu, Jiang, Huang, Chang, Wu, Roffler and Yeh (1995) *Eur. J. Cancer* **31**, 237–243]. In this study, when treated with RA, SC-M1/Bcl2 cells, which were generated by transfecting SC-M1 cells with bcl-2 DNA, were growth-arrested two days earlier than SC-M1/neo cells, which were generated by transfecting SC-M1 cells with vector DNA. This indicates that Bcl-2 accelerates RA-induced growth arrest. In addition to the accelerated growth arrest, RA-treated SC-M1/Bcl2 cells also recovered from growth arrest two days faster than SC-M1/neo cells after the removal of RA. Previously, we had identified the cyclin-dependent kinase inhibitor p21^(WAF1/CIP1)

(p21) as a mediator of RA-induced growth arrest [Tsao, Li, Kuo, Liu and Chen (1996) *Biochem. J.* **317**, 707–711]. In a search for the mechanism by which Bcl-2 affects growth regulation, we found that p21 gene expression was more prominent in SC-M1/Bcl2 cells than in SC-M1/neo cells in the presence of RA, but when RA was removed, p21 gene expression levels in SC-M1/Bcl2 cells were also reduced earlier than in SC-M1/neo cells. The present report is the first to show that Bcl-2 accelerates not only growth arrest but also recovery from growth arrest. Moreover, the close correlation between the effect of Bcl-2 on both RA-induced growth arrest and RA-induced p21 gene expression suggests the possibility that Bcl-2 affects cell growth through the mechanism of p21.

Key words: Bcl-2, growth arrest, p21, SC-M1 cells.

INTRODUCTION

Bcl-2 is the first negative regulator of apoptotic cell death to be identified [1–3]. Apoptosis is an active form of cell suicide that is associated with morphological changes such as cell shrinkage, cell membrane blebbing, nuclear fragmentation and DNA degradation [4]. The exogenous expression of the bcl-2 gene is shown to protect cells from apoptosis induced by p53 overexpression and various stimuli, such as growth-factor withdrawal, irradiation and chemotherapy [1,5–6]. Those cells which survive with Bcl-2 protection against environmental stimuli generally undergo cell-cycle arrest [2,5–6]. There is other recent evidence suggesting that Bcl-2 may also affect growth regulation. For example, it has been shown that Bcl-2 delays the entry into the cell cycle of mitogen-stimulated B and T lymphocytes and delays the serum stimulation-induced entry into S phase of quiescent NIH3T3 fibroblasts [7]. Moreover, certain evidence *in vivo* also indicates the involvement of Bcl-2 in growth regulation. For example, a high concentration of Bcl-2 can be detected in premalignant stomach lesions but not in stomach carcinoma [8]. In addition, expression of Bcl-2 is reported to inversely correlate with the mitotic index of stomach lesions [9,10]. In such situations, it is possible that Bcl-2 might prevent malignant transformation by inhibiting cell growth *in vivo*. These facts indicate that Bcl-2 may inhibit growth when normal cell growth is perturbed.

Retinoic acid (RA)-inhibited growth of SC-M1 cells can be maintained for 70 days by continuous RA exposure, and remains reversible after the removal of RA [11–13]. This provides an excellent system to study mechanisms involved in growth regu-

lation. To further establish its reversible growth inhibitory effect, Bcl-2 was tested on SC-M1 gastric cancer cells. Therefore, in the present study, we investigated whether Bcl-2 could regulate growth of SC-M1 cells in the presence of RA. We found not only that Bcl-2 did indeed accelerate RA-induced growth arrest but also that an enhanced RA-induced p21 [cyclin-dependent kinase inhibitor p21^(WAF1/CIP1)] expression in Bcl-2 containing cells was observed, suggesting that p21 mediated the early growth arrest of Bcl-2-containing SC-M1 cells. A surprising result of the study was the faster recovery of RA-treated SC-M1/Bcl2 cells from growth arrest after the removal of RA when compared with the recovery of the RA-treated control cells (SC-M1/neo); furthermore, the decrease in p21 gene expression was faster than that of SC-M1/neo cells. These results indicate that Bcl-2 accelerated both growth arrest and recovery.

EXPERIMENTAL

Cells

SC-M1, a gastric cancer cell line [14] (kindly provided by Dr. Ching-Liang Meng), was cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco Laboratories, Grand Island, NY, U.S.A.) containing 10% (v/v) fetal-bovine serum (FBS; Gibco) and 100 units/ml penicillin and streptomycin. SC-M1 cells were transfected with a pCEP4-based plasmid (Invitrogen, Carlsbad, CA, U.S.A.) encoding human bcl-2 cDNA. After selection with 200 µg/ml hygromycin for 3 weeks, resistant clones were pooled, and named SC-M1/Bcl2.

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; FBS, fetal-bovine serum; p21, cyclin-dependent kinase inhibitor p21^(WAF1/CIP1); RA, retinoic acid; m.o.i., multiplicity of infection.

¹ To whom correspondence should be addressed (e-mail yptsao@kimo.com.tw).

Cell proliferation assay

Cells were plated on to a 6-well plate at a density of 3×10^4 cells per well in DMEM containing 10% (v/v) FBS and 100 units/ml penicillin and streptomycin. After 24 h, the medium was changed to DMEM with 10% (v/v) FBS in the presence of 5 μ M RA or an equal amount of DMSO. The cell numbers were counted every other day using a haemocytometer. Viability was determined by Trypan Blue exclusion.

RNA isolation and Northern-blot analysis

The single-step method of RNA isolation by acid guanidinium thiocyanate/phenol/chloroform (1:1:0.2, by vol.) extraction was used to obtain total cell RNA. The RNA was separated by electrophoresis on a formaldehyde/1% agarose denaturing gel and then transferred to a nitrocellulose filter. The filters were then hybridized with probes at 68 °C as follows. The hybridization buffer contained 50% (v/v) formamide, 5 $\times$ Denhardt's solution, 5 $\times$ SSPE (where 1 $\times$ SSPE is 0.15 M NaCl/10 mM NaH₂PO₄/1 mM EDTA, pH 7.4), 10% (w/v) dextran sulphate, 0.1% (w/v) SDS and 200 μ g/ml denatured salmon sperm DNA. The probes were p21 cDNA (kindly provided by Dr David Beach, Cold Spring Harbor Laboratory, NY, U.S.A.) and β -actin cDNA labelled with [α -³²P]dCTP by nick translation. After hybridization, the filters were washed in 0.1 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl/0.015 M sodium citrate) containing 0.1% (w/v) SDS at 60 °C, and then air-dried and autoradiographed at -70 °C. The intensity of the bands in the autoradiograms was measured by laser densitometry.

Immunoblot

Cellular proteins were extracted into SDS/PAGE sample buffer (New England Biolabs) and boiled for 10 min. The crude protein lysates (150 μ g) were separated by SDS/PAGE, transferred to a nitrocellulose filter, reacted with specific primary antibodies and were revealed using secondary antibodies and Western Blue stabilized for alkaline phosphatase (Promega, Madison, WI, U.S.A.). Antibodies recognizing p21 and Bcl-2 were purchased from Pharmingen (San Diego, CA, U.S.A.). The gels were subjected to autoradiography and the intensity of the bands was estimated by laser densitometry.

Flow cytometric analysis of DNA content

Cells were treated with trypsin and resuspended at 5×10^6 cells/ml in citrate buffer [250 mM sucrose/40 mM sodium citrate/5% (v/v) DMSO, pH 7.6]. A 200 μ l sample was added to 1.8 ml of solution A [30 μ g/ml trypsin, 3.4 mM sodium citrate, 0.1% (v/v) Nonidet P40, 1.5 mM spermine and 0.5 mM Tris/HCl, pH 7.6] and incubated for 20 min at room temperature. Following this, 1.5 ml of solution B [500 μ g/ml trypsin inhibitor (Sigma), 100 μ g/ml ribonuclease A (Sigma), 3.4 mM sodium citrate, 0.1% Nonidet P40, 1.5 mM spermine and 0.5 mM Tris/HCl, pH 7.6] was added and the solution was incubated for 20 min at room temperature. Finally 1.5 ml of ice-cold solution C (416 μ g/ml propidium iodide, 3.4 mM sodium citrate, 0.1% Nonidet P40, 1.5 mM spermine and 0.5 mM Tris/HCl, pH 7.6) was added and the incubation was continued at 4 °C for 20 min. Flow cytometric analysis was performed using a FACScan (Beckton and Dickinson). Analysis of the percentage of cells in the S phase was performed using CellFIT cell-cycle analysis software (Beckton and Dickinson).

Statistical analysis

The *P* values were calculated by the Wilcoxon rank-sum test. A significant correlation between two parameters was taken at the 95% confidence limit, where *P* < 0.05.

RESULTS

Establishment of a variant of SC-M1 overproducing Bcl-2

To investigate the effect of Bcl-2 on RA-induced growth arrest in SC-M1 cells, SC-M1/Bcl2 cells were established by transfecting bcl-2 cDNA into SC-M1 cells and then by pooling at least 80 hygromycin-resistant colonies. As a control, vector plasmid (pCEP4; Invitrogen) was transfected into SC-M1 cells to establish SC-M1/neo cells. As shown in Figure 1, high levels of Bcl-2 protein in SC-M1/Bcl2 cells were detected by immunoblotting. This cell line was used to study the effect of Bcl-2 on RA-induced growth arrest, since Bcl-2 levels were not affected by exposure of the cells to 5 μ M RA for 2 days. The growth rates of cells revealed that SC-M1/Bcl2 cells proliferated at a similar rate to SC-M1 and SC-M1/neo cells in the absence of RA. The cell growth of SC-M1/Bcl2 cells under the influence of RA was moderately slower than that of SC-M1/neo cells (Figure 2).

Bcl-2 accelerates RA-induced growth arrest of SC-M1 cells

To investigate the effect of Bcl-2 on RA-induced growth arrest, flow cytometric analysis of DNA contents in SC-M1/Bcl2 and SC-M1/neo cells in the presence or absence of RA treatment was performed. As shown in Table 1, the percentage of cells in S phase, which represents the status of cell growth, remained around 26–35% in both SC-M1/Bcl2 cells and SC-M1/neo cells in the absence of RA treatment during the 6-day culture period. RA exposure reduced the percentage of cells in S phase to 11.2% in 2 days in SC-M1/Bcl2 cells. However, RA exposure for 4 days

Figure 1 Expression of Bcl-2 by Western-blot analysis

Cell lysates (150 μ g) from SC-M1, SC-M1/neo and SC-M1/Bcl2 cells in the presence or absence of 5 μ M RA for 2 days were extracted and analysed by SDS/PAGE. Immunoblot analysis was then performed using antibodies recognizing Bcl-2. β -Actin-specific antibodies were used to ensure that equal amounts of protein were present in different lanes. A representative autoradiogram from three independent experiments is shown.

Figure 2 Cell growth of SC-M1, SC-M1/neo and SC-M1/Bcl2 cells in response to RA exposure

Cells (3×10^4) were plated on to a 6-well plate and grown in the presence or absence of $5 \mu\text{M}$ RA. Cell numbers were counted every 2 days using a haemocytometer.

was necessary to reduce the percentage of cells in S phase to a similar level (13.4%) in SC-M1/neo cells. The difference in the decrease of the S phase percentage for longer exposure (such as 6 days) of both SC-M1/Bcl2 and SC-M1/neo cells to RA was not significant. In summary, these observations indicate that the response of Bcl-2-containing cells to RA-induced growth arrest is faster than that of SC-M1/neo cells. In addition, since Bcl-2 has an anti-apoptotic effect [1–3], we examined apoptosis of SC-M1/Bcl2 and SC-M1/neo before and after exposure to RA to study what function the anti-apoptotic protein might have in growth arrest regulation. Even with overexpressed exogenous *Bcl-2* gene, the SC-M1/Bcl2 cells had the same ratio of apoptosis as the SC-M1/neo cells (approx. 1%) when measured using the annexin V apoptosis assay. The ratio remained the same in both cell lines when subjected to RA exposure (results not shown).

Table 2 Flow cytometric analysis of DNA content of SC-M1/neo and SC-M1/Bcl2 cells after the removal of RA

Exponential phase SC-M1/neo and SC-M1/Bcl2 cells were cultured in the presence of $5 \mu\text{M}$ RA for 6 days. RA was then removed by washing the cells twice in PBS, and the culture medium was changed. At 4, 6 and 8 days after the removal of RA, cells were treated as described in the Experimental section and subjected to flow cytometric analysis of DNA content. The results are the percentage ($\pm$ S.D.) of cells in each phase of the cell cycle from three separate experiments.

Days after removal of RA ...	Percentage of cells in each phase		
	4	6	8
SC-M1/neo cells			
G ₀ /G ₁	87.3 $\pm$ 1.1	81.2 $\pm$ 1.1	72.1 $\pm$ 1.0
S	11.7 $\pm$ 0.2	12.3 $\pm$ 0.2	18.6 $\pm$ 0.3
G ₂ /M	1.0 $\pm$ 0.3	6.5 $\pm$ 0.3	9.3 $\pm$ 0.5
SC-M1/Bcl2 cells			
G ₀ /G ₁	87.4 $\pm$ 1.3	77.3 $\pm$ 1.1	67.2 $\pm$ 1.1
S	10.6 $\pm$ 0.3	22.7 $\pm$ 0.4	31.7 $\pm$ 1.2
G ₂ /M	2.0 $\pm$ 0.5	0	1.1 $\pm$ 0.4

This observation suggests that Bcl-2 does not function as an anti-apoptotic protein in SC-M1 cells in the presence of RA.

Bcl-2 accelerates the reversal of RA-induced growth arrest

One of the possible explanations for the Bcl-2-accelerated RA-induced growth arrest is that Bcl-2 itself may have a growth-suppression effect. The acceleration of growth arrest may represent the synergistic effect of RA and Bcl-2. Under this assumption, Bcl-2 would be expected to slow down the recovery from growth arrest after the removal of RA. SC-M1/neo and SC-M1/Bcl2 cells were exposed to RA for 6 days to achieve complete growth arrest. After the removal of RA cell growth was assayed by monitoring the S-phase population of cells by flow cytometry. Surprisingly, the results showed that Bcl-2-expressing cells (SC-M1/Bcl2) recovered faster than SC-M1/neo cells from growth arrest (Table 2). The percentage of cells in S phase was greater in SC-M1/Bcl2 cells (22.7% at 6 days and 31.7% at 8 days) than in SC-M1/neo cells (12.3% at 6 days and 18.6% at 8 days) after the removal of RA. Since SC-M1/Bcl2 cells recover more quickly from RA-induced growth arrest, Bcl-2 may have a stimulatory effect on growth when RA is removed. These observations indicated the potential role of Bcl-2 as a bi-

Table 1 Flow cytometric analysis of SC-M1/neo cells and SC-M1/Bcl2 cells after RA exposure

Exponential-phase SC-M1/neo and SC-M1/Bcl2 cells were cultured in the presence of $5 \mu\text{M}$ RA or an equal volume of DMSO (0) for 2, 4 or 6 days respectively. Cells were then treated as described in the Experimental section and subjected to flow cytometric analysis of DNA content. The results are the percentages ($\pm$ S.D.) of cells in each phase of the cell cycle from three separate experiments.

RA (μM) ...	Percentage of cells in each phase					
	0	4	6	5	4	6
Culture time (days) ...	2			2		
SC-M1/neo cells						
G ₀ /G ₁	62.4 $\pm$ 1.2	67.5 $\pm$ 1.3	66.3 $\pm$ 1.3	75.6 $\pm$ 1.2	85.6 $\pm$ 1.2	87.2 $\pm$ 1.0
S	35.2 $\pm$ 1.1	30.4 $\pm$ 0.5	25.4 $\pm$ 0.2	23.3 $\pm$ 0.4	13.4 $\pm$ 0.3	8.2 $\pm$ 0.3
G ₂ /M	2.4 $\pm$ 0.1	2.1 $\pm$ 0.1	8.3 $\pm$ 0.5	1.1 $\pm$ 0.2	1.0 $\pm$ 0.1	4.6 $\pm$ 0.2
SC-M1/Bcl2 cells						
G ₀ /G ₁	62.6 $\pm$ 1.3	68.6 $\pm$ 0.5	69.5 $\pm$ 1.3	83.2 $\pm$ 1.6	83.5 $\pm$ 1.4	87.5 $\pm$ 1.1
S	35.1 $\pm$ 1.2	30.3 $\pm$ 1.0	26.4 $\pm$ 0.7	11.2 $\pm$ 0.5	10.6 $\pm$ 0.4	7.8 $\pm$ 0.3
G ₂ /M	2.3 $\pm$ 0.2	1.1 $\pm$ 0.1	4.1 $\pm$ 0.4	5.6 $\pm$ 0.4	5.9 $\pm$ 0.5	4.7 $\pm$ 0.5

Figure 3 RA treatment increases the concentrations of p21 protein and RNA in SC-M1/Bcl2 cells when compared with SC-M1/neo cells

Exponential-phase growing SC-M1/neo and SC-M1/Bcl2 cells were cultured in the presence of 5 μ M RA or an equal volume of DMSO for the indicated times. (A) Cell lysates were extracted and separated by SDS/PAGE. Immunoblot analysis was then performed using antibodies recognizing p21. A representative autoradiogram from four independent experiments is shown. (B) Northern blot analysis. Total cellular RNA was extracted, separated on formaldehyde/agarose (1.0% gel), blotted on to a nitrocellulose filter and hybridized to p21-specific cDNA probes, as described in the Experimental section. The filter was then hybridized to β -actin-specific cDNA probes to ensure that equal amounts of RNA were present in the different lanes. A representative autoradiogram from four independent experiments is shown. *Significant difference between fold change in SC-M1/Bcl2 compared with SC-M1/neo cells at $P < 0.05$ by Wilcoxon rank-sum test.

directional catalyst to accelerate RA-induced growth regulation, both in growth arrest and recovery from growth arrest.

Bcl-2 enhances p21 induction by RA treatment

We further studied the mechanism by which Bcl-2 accelerated RA-induced growth arrest. Since p21 is proposed to be involved in this phenomenon [15–18], we investigated whether Bcl-2 overexpression raised the concentration of p21 during RA-

Figure 4 Concentrations of p21 protein and RNA after removal of RA treatment

Exponential-phase growing SC-M1/neo cells and SC-M1/Bcl2 cells were cultured in the presence of 5 μ M RA for 6 days. RA was then removed by changing the medium. (A) Cell lysates were extracted at the indicated time points and analysed by immunoblotting with antibodies recognizing p21. A representative autoradiogram from four independent experiments is shown. (B) Total cellular RNA was extracted at the indicated time points, separated on formaldehyde/agarose (1.0% gel), blotted on to a nitrocellulose filter and hybridized to a p21-specific cDNA probe, as described in the Experimental section. The filter was then hybridized to a β -actin-specific cDNA probe to ensure that equal amounts of RNA were present in different lanes. A representative autoradiogram from four independent experiments is shown. *Significant difference between fold change in SC-M1/Bcl2 compared with SC-M1/neo cells at $P < 0.05$ by Wilcoxon rank-sum test.

induced growth arrest. Immunoblot analysis revealed greater p21 protein concentrations in SC-M1/Bcl2 cells than in SC-M1/neo cells after RA exposure (Figure 3A). The concentrations

Figure 5 Exogenous p21 induces growth arrest both in SC-M1/neo and SC-M1/Bcl2 cells

Exponential-phase growing SC-M1/neo and SC-M1/Bcl2 cells were infected with recombinant adenovirus encoding *lacZ* and *p21* at m.o.i. of 100. (A) Cell lysates (150 μ g) were extracted after 48 h and analysed by immunoblotting p21 protein. A representative autoradiogram from three independent experiments is shown. (B) Cell morphology of: (a) SC-M1/neo cells infected with recombinant adenovirus encoding *lacZ* for 2 days, (b) SC-M1/neo cells infected with recombinant adenovirus encoding *p21* for 2 days, (c) SC-M1/Bcl2 cells infected with *lacZ*-encoded adenovirus for 2 days, and (d) SC-M1/Bcl2 cells infected with *p21*-encoded adenovirus for 2 days.

of p21 protein after exposure to RA for 2, 4 or 6 days in SC-M1/Bcl2 cells were 1.7-, 1.4- and 1.2-fold of those in SC-M1/neo cells respectively. Whereas the comparative levels of p21 protein in SC-M1/Bcl2 increased significantly within 2 days of RA treatment, Northern-blot analysis showed that, during the first 3 days, p21 mRNA levels were also significantly higher in SC-M1/Bcl2 cells (1.7-, 1.7- and 1.6-fold) than in SC-M1/neo cells after exposure to RA, when assessed using Wilcoxon rank-sum test. By day 4 of exposure, the levels of mRNA reached a plateau and the concentrations of p21 mRNA were not significantly

Table 3 Flow cytometric analysis of DNA content of SC-M1/neo and SC-M1/Bcl2 cells infected with adenovirus/*lacZ* or adenovirus/*p21*

Exponential phase SC-M1/neo and SC-M1/Bcl2 cells were infected with adenovirus/*lacZ* or adenovirus/*p21* at a m.o.i. of 100 for 48 h. The cells were treated as described in the Experimental section and subjected to flow cytometric analysis of DNA content. The results are the percentages ($\pm$ S.D.) of cells in each phase of the cell cycle from three separate experiments.

	Percentage of cells in each phase	
	Adenovirus/ <i>lacZ</i>	Adenovirus/ <i>p21</i>
SC-M1/neo cells		
G ₀ /G ₁	62.8 $\pm$ 1.2	80.3 $\pm$ 1.5
S	35.6 $\pm$ 1.0	18.7 $\pm$ 0.7
G ₂ /M	1.6 $\pm$ 0.3	1.0 $\pm$ 0.1
SC-M1/Bcl2		
G ₀ /G ₁	68.2 $\pm$ 1.4	83.1 $\pm$ 1.1
S	29.8 $\pm$ 1.2	14.8 $\pm$ 0.5
G ₂ /M	2.0 $\pm$ 0.2	2.1 $\pm$ 0.4

different (Figure 3B). Taken together, these results suggest that Bcl-2 may amplify RA-induced *p21* gene induction during growth arrest.

As our previous observations indicated that Bcl-2 accelerated the recovery from growth arrest after the removal of RA (Table 2), we also investigated the p21 levels in SC-M1/neo and SC-M1/Bcl2 cells after the removal of RA using immunoblot and Northern-blot analysis. The levels of p21 protein (Figure 4A) and p21 RNA (Figure 4B) fell more quickly in SC-M1/Bcl2 cells than in SC-M1/neo cells. The concentrations of p21 protein in SC-M1/Bcl2 cells decreased to 0.5-, 0.2- and 0.3-fold of the SC-M1/neo cells after the removal of RA for 4, 6 and 8 days respectively. The difference was significant by Wilcoxon rank-sum test. Similarly, the levels of p21 mRNA also fell more quickly in SC-M1/Bcl2 cells than in SC-M1/neo cells (0.5-, 0.2- and 0.6-fold) (Figure 4B). These results suggest that Bcl-2 may accelerate the recovery of growth arrest by repressing *p21* gene expression after the removal of RA.

p21 is a mediator of RA-induced growth arrest

After documenting the correlation of the effect of Bcl-2 on RA-induced growth arrest and RA-induced *p21* gene expression, the next step was to further establish p21 as the mediator of growth arrest in SC-M1/neo and SC-M1/Bcl2 cells. We tested the capability of p21 to arrest cell growth by overexpressing the exogenous *p21* gene in the two cell lines. SC-M1/neo and SC-M1/Bcl2 cells were transduced by an adenovirus encoding the *p21* gene at 100 multiplicity of infection (m.o.i.) [19]. Figure 5(A) shows high p21 protein expression in cells infected with recombinant adenovirus encoding p21, but not in virus encoded with the *lacZ* gene. Two days after virus p21 infection, both SC-M1/neo (Figure 5B, panel b) and SC-M1/Bcl2 cells (Figure 5B, panel d) acquired the flattened morphological changes showing growth arrest. The same morphological phenomenon occurred when SC-M1/neo cells and SC-M1/Bcl2 cells were exposed to RA (results not shown). Figure 5(B) (panels a and c) show the lack of morphological change in both the SC-M1/neo and the SC-M1/Bcl2 cells when each was infected with an equivalent titre of recombinant adenovirus encoding *lacZ*. As shown in Table 3, flow cytometric analysis of p21 adenovirus-infected cells

revealed the depletion of S phase cells 2 days after infection. After adenovirus-p21 infection, the number of S-phase cells in SC-M1/neo cells was reduced to 18.7% compared with 35.6% of cells in S phase after infection with adenovirus-lacZ. The ability of exogenous p21 to achieve a similar effect on cell-growth arrest to that of RA, together with the correlation between RA-induced growth arrest and *p21* gene induction, provided some, but not sufficient, evidence for the possible role of p21 as a mediator of the growth arrest induced by RA in both SC-M1/neo and SC-M1/Bcl2 cells.

DISCUSSION

RA is reported to induce cell-growth arrest and cell differentiation in experimental and physiological conditions [20–22]. Indeed, the induction of cell differentiation by RA has been widely used for elucidating the regulating mechanism of cell growth and differentiation. Growth arrest by RA in most research models is irreversible [20–22], this makes the correlation of the biological response with growth regulation or differentiation difficult. However, SC-M1 cells offer a unique system by which RA treatment can induce reversible growth arrest and morphological changes [11–13]. Any biological event can be better correlated with RA-induced growth arrest if it appears with RA treatment and disappears after the removal of RA. In the present study, p21, which correlated well with the reversible cell-growth arrest, may also participate in the growth arrest induced by RA (Figure 4). A supplementary piece of evidence came from the finding that overexpression of p21 by an exogenous *p21* gene delivered by an adenoviral vector led to growth arrest in SC-M1 cells (Table 3).

It was also found that SC-M1/Bcl2 cells, when treated with RA, were arrested in growth 2 days earlier than SC-M1/neo cells (Table 1), indicating that Bcl-2 accelerates RA-induced growth arrest. Further, in addition to this accelerated growth arrest, RA-treated SC-M1/Bcl2 cells also recovered from growth arrest after the removal of RA 2 days faster than SC-M1/neo cells (Table 2). The present report is the first to demonstrate that Bcl-2 accelerates not only growth arrest but also recovery from growth arrest.

In the present study, it was found that Bcl-2 overexpression did not affect cell growth (Figure 2). However, in a previous study, it was stated that the colorectal adenocarcinoma cell line (SW480) was the only cell line among several tested where growth was inhibited by Bcl-2 [23]. This observation was measured by transfecting Bcl-2 cDNA into SW480 cells. Colony formation was counted by drug selection. It is not known if growth arrest was the mechanism behind the failure of colony formation. It is, nevertheless, still reasonable to assume that Bcl-2 does not affect the growth of normal cells.

In a search for the mechanism of growth regulation by Bcl-2, in the present study, it was found that the *p21* gene product was an important factor in growth arrest induced by RA, whereas Bcl-2 moderately enhanced the induction of p21 in the presence of RA (Figure 3). This suggests a model where Bcl-2 is involved in RA-induced growth arrest by enhancing the expression of the *p21* gene. It is unlikely that Bcl-2 directly affects *p21* gene expression since Bcl-2 is not a nuclear protein, and has never been reported to be a transcription factor. However, overexpression of Bcl-2 is shown to induce apoptosis-associated changes, such as alteration of the cellular redox state [24–27], change in subcellular calcium ion distribution [28,29], activation of caspases [30] and distribution of the mitochondrial membrane potential [31–33]. These mechanisms may be involved in the regulation of *p21* gene expression resulting from the presence of Bcl-2. Interestingly, the redox state is reported to mediate the regulation of *p21* gene expression [34]. The mecha-

nisms behind the regulation of *p21* gene expression by Bcl-2 are currently being investigated.

From the involvement of Bcl-2 in the growth arrest process, as described above, it is reasonable to conclude that Bcl-2 accelerates RA-induced growth arrest. However, in the present study, by removing RA, we also found that *Bcl-2* transgene cells recovered more quickly from RA-induced growth arrest and regained growth potential faster than the control cells. This suggests that the growth potential of cells can be determined by RA treatment but not by Bcl-2. Our findings that Bcl-2 enhances not only RA-induced growth arrest but also recovery from growth arrest, provide an excellent model with which to study the effects of Bcl-2 on growth regulation. Unravelling the conditions and mechanisms by which Bcl-2 regulates cell growth may advance our knowledge of tumour formation and cancer therapy.

We are grateful to Dr David Beach for providing p21 cDNA, and Mr John Wu, Mr Daniel L. Altschuler and Mr Thomas Price for their help in modifying the English of this manuscript. This work was supported by National Science Council Grants, NSC 87-2312-B016-004.

REFERENCES

- Vaux, D. L., Cory, S. and Adams, J. M. (1988) Bcl-2 gene promotes haemopoietic cell survival and cooperates with c-myc to immortalize pre-B cells. *Nature (London)* **335**, 440–442.
- Hockenbery, D. M., Nunez, G., Millman, C., Schreiber, R. D. and Korsmeyer, S. J. (1990) Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. *Nature (London)* **348**, 334–336.
- Kroemer, G. (1997) The proto-oncogene Bcl-2 and its role in regulating apoptosis. *Nat. Med.* **3**, 614–620.
- Williams, G. T. (1991) Programmed cell death: Apoptosis and oncogenesis. *Cell* **65**, 1097–1098.
- Miyashita, T. and Reed, J. C. (1995) Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. *Cell* **80**, 293–299.
- Chiou, S. K., Rao, L. and White, E. (1994) Bcl-2 blocks p53-dependent apoptosis. *Mol. Cell. Biol.* **14**, 2556–2563.
- O'Reilly, L. A., Huang, D. C. S. and Strasser, A. (1996) The cell death inhibitor Bcl-2 and its homologues influence control of cell cycle entry. *EMBO J.* **15**, 6979–6990.
- Nakamura, T., Nomura, S., Sakai, T. and Nariya, S. (1997) Expression of bcl-2 oncoprotein in gastrointestinal and uterine carcinomas and their premalignant lesions. *Hum. Pathol.* **28**, 309–315.
- Uesugi, H., Saegusa, M., Takano, Y. and Okayasu, I. (1996) Different expression of Bcl-2 protein in gastric adenomas and carcinomas. *Pathol. Int.* **46**, 274–280.
- Koshida, Y., Saegusa, M. and Okayasu, I. (1997) Apoptosis, cell proliferation and expression of Bcl-2 and Bax in gastric carcinomas: immunohistochemical and clinicopathological study. *Br. J. Cancer* **75**, 367–373.
- Shyu, R. Y., Jiang, S. Y., Huang, S. L., Chang, T. C., Wu, K. L., Roffler, S. R. and Yeh, M. Y. (1995) Growth regulation by all-trans-retinoic acid and retinoic acid receptor messenger ribonucleic acids expression in gastric cancer cells. *Eur. J. Cancer* **31**, 237–243.
- Jiang, S. Y., Shyu, R. Y., Chen, H. Y., Lee, M. M., Wu, K. L. and Yeh, M. Y. (1996) *In vitro* and *in vivo* growth inhibition of SC-M1 gastric cancer cells by retinoic acid. *Oncology* **53**, 334–340.
- Chao, T. Y., Jiang, S. Y., Shyu, R. Y., Yeh, M. Y. and Chu, T. M. (1997) All-trans retinoic acid decreases susceptibility of a gastric cancer cell line to lymphokine-activated killer cytotoxicity. *Br. J. Cancer* **75**, 1284–1290.
- Tzeng, C. C., Meng, C. L., Jin, L. and Hsieh, H. F. (1991) Cytogenetic studies of gastric adenocarcinoma. *Cancer Genet. Cytogenet.* **55**, 67–71.
- Tsao, Y. P., Li, S. F., Kuo, S. W., Liu, J. C. and Chen, S. L. (1996) Reversal of the temperature-shift-induced growth restriction of a temperature-sensitive simian virus 40 T-antigen-transformed human fibroblast cell line by treatment with retinoic acid. *Biochem. J.* **317**, 707–711.
- Liu, M., Iavarone, A. and Freedman, L. P. (1996) Transcriptional activation of the human p21(WAF1/CIP1) gene by retinoic acid receptor. Correlation with retinoid induction of U937 cell differentiation. *J. Biol. Chem.* **271**, 31723–31728.
- Naka, K., Yokozaki, H., Domen, T., Hayashi, K., Kuniyasu, H., Yasui, W., Lotan, R. and Tahara, E. (1997) Growth inhibition of cultured human gastric cancer cells by 9-cis-retinoic acid with induction of cdk inhibitor Waf1/Cip1/Sd1/p21 protein. *Differentiation* **61**, 313–320.

- 18 Hsu, C. A., Rishi, A. K., Su-Li, X., Gerald, T. M., Dawson, M. I., Schiffer, C., Reichert, U., Shroot, B., Poirer, G. C. and Fontana, J. A. (1997) Retinoid induced apoptosis in leukemia cells through a retinoic acid nuclear receptor-independent pathway. *Blood* **89**, 4470–4479
- 19 Tsao, Y. P., Huang, S. J., Hsieh, J. T., Chang, J. L., Pong, R. C. and Chen, S. L. (1999) Adenovirus-mediated p21 (WAF1/SDI1/CIP1) gene transfer induces apoptosis of human cervical cancer cell lines. *J. Virol.* **73**, 4983–4990
- 20 Lotan, R., Lotan, D. and Sacks, P. G. (1990) Inhibition of tumor cell growth by retinoids. *Methods Enzymol.* **190**, 100–110
- 21 Smith, M. A., Parkison, D. R., Cheson, B. D. and Friedman, M. A. (1992) Retinoids in cancer therapy. *J. Clin. Oncol.* **10**, 839–864
- 22 Gudas, L. J. (1992) Retinoids, retinoid-responsive genes, cell differentiation, and cancer. *Cell Growth Differ.* **3**, 655–662
- 23 Pietenpol, J. A., Papadopoulos, N., Markowitz, S., Willson, J. K. V., Kinzler, K. W. and Vogelstein, B. (1994) Paradoxical inhibition of solid tumor cell growth by bcl2. *Cancer Res.* **54**, 3714–3717
- 24 Hockenbery, D. M., Oltvai, Z. N., Yin, X. M., Millman, C. L. and Korsmeyer, S. J. (1993) Bcl-2 functions in an antioxidant pathway to prevent apoptosis. *Cell* **75**, 241–251
- 25 Kane, D. J., Sarafian, T. A., Anton, R., Hahn, H., Gralla, E. B., Valentine, J. S., Ord, T. and Bredesen, D. E. (1993) Bcl-2 inhibition of neural death: decreased generation of reactive oxygen species. *Science* **262**, 1274–1277
- 26 Lawrence, M. S., Ho, D. Y., Sun, G. H., Steinberg, G. K. and Sapolsky, R. M. (1996) Overexpression of Bcl-2 with herpes simplex virus vectors protects CNS neurons against neurological insults *in vitro* and *in vivo*. *J. Neurosci.* **16**, 486–496
- 27 Ellerby, L. M., Ellerby, H. M., Park, S. M., Holleran, A. L., Murphy, A. N., Fiskum, G., Kane, D. J., Testa, M. P., Kayalar, C. and Bredesen, D. E. (1996) Shift of the cellular oxidation-reduction potential in neural cells expressing Bcl-2. *J. Neurochem.* **67**, 1259–1267
- 28 Lam, M., Dubyak, G., Chen, L., Nunez, G., Miesfeld, R. L. and Distelhorst, C. W. (1994) Evidence that BCL-2 represses apoptosis by regulating endoplasmic reticulum-associated Ca^{2+} fluxes. *Proc. Natl. Acad. Sci. U.S.A.* **91**, 6569–6573
- 29 Marin, M. C., Fernandez, A., Bick, R. J., Brisbay, S., Buja, L. M., Snuggs, M., McConkey, D. J., von Eschenbach, A. C., Keating, M. J. and McDonnell, T. J. (1996) Apoptosis suppression by bcl-2 is correlated with the regulation of nuclear and cytosolic Ca^{2+} . *Oncogene* **12**, 2259–2266
- 30 Chinnaiyan, A. M., Orth, K., O'Rourke, K., Duan, H., Poirier, G. G. and Dixit, V. M. (1996) Molecular ordering of the cell death pathway. Bcl-2 and Bcl-xL function upstream of the CED-3-like apoptotic proteases. *J. Biol. Chem.* **271**, 4573–4576
- 31 Kroemer, G., Petit, P., Zamzami, N., Vayssiere, J. L. and Mignotte, B. (1995) The biochemistry of programmed cell death. *FASEB J.* **9**, 1277–1287
- 32 Yang, J., Liu, X., Bhalla, K., Kim, C. N., Ibrado, A. M., Cai, J., Peng, T. I., Jones, D. P. and Wang, X. (1997) Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked. *Science* **275**, 1129–1132
- 33 Kluck, R. M., Bossy-Wetzel, E., Green, D. R. and Newmeyer, D. D. (1997) The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis. *Science* **275**, 1132–1136
- 34 Esposito, F., Cuccovillo, F., Vanoni, M., Cimino, F., Anderson, C. W., Appella, E. and Russo, T. (1997) Redox-mediated regulation of p21(waf1/cip1) expression involves a post-transcriptional mechanism and activation of the mitogen-activated protein kinase pathway. *Eur. J. Biochem.* **245**, 730–737

Received 6 December 1999/14 March 2000; accepted 5 April 2000