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Comparative Study of Anti-Apoptotic Genes, *Bcl*-2 and *P35* for the Suppression of Apoptosis Induced in Suspension Culture of Transformed *Trichoplusia ni* BTI Tn 5B1-4 Cells

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To delay the onset of apoptosis in the culture, transformed Tn 5B1-4 cells harboring anti-apoptotic genes, *bcl*-2 and baculovirus *p35*, have been established and analyzed for their anti-apoptotic ability in suspension culture using spinner flasks. In the suspension culture at agitation speeds of 100 rpm and 200 rpm, the cell growth of cell clone expressing *Bcl*-2 protein was much higher than other two clones and the maximum cell density of the clone was 6.0 × 10^6 cells/ml and 6.2 × 10^6 cells/ml at day three of the incubation. On the other hand, the cell growth of cell clone expressing baculovirus protein *P35* was much higher than other two clones in suspension culture at agitation speed of 300 rpm and the maximum cell density of the clone was 6.1 × 10^6 cells/ml at day three of the incubation. Based on the pattern of genomic DNA laddering and the microscopic observation of apoptotic bodies, the more apoptotic bodies are induced in Tn 5B1-4 control cell clone at higher agitation speed. This result shows that the shear stress can be a main factor in inducing apoptosis in spinner flask culture. At low agitation speed, cell clone expressing *Bcl*-2 was more effective in delaying the onset of apoptosis than the cell clone expressing *P35*. On the other hand, at high agitation speed, cell clones expressing baculovirus *P35* was more effective in delaying the onset of apoptosis than the cell clone expressing *Bcl*-2. Therefore, anti-apoptotic genes, *bcl*-2 and baculovirus *p35*, can play a distinct role depending on agitation speed in the suspension culture.

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Introduction

Apoptotic cells follow a progressive morphological change that is characterized by plasma-membrane blebbing, cell shrinkage, chromatin condensation, as well as nuclear degradation, and results in fragmentation into apoptotic bodies (Wyllie *et al.*, 1980). This effector/degradation phase is common to all apoptotic processes. The family of cysteine aspartate proteases, or caspases, currently comprises ten different members, and constitutes one of the most important classes of gene products acting in this process. In the two major mammalian apoptotic pathways, the mitochondria pathway and the cell surface death receptor pathway, caspase-9 and caspase-8 are two key initiator caspases that can be, respectively, activated when cellular or environmental apoptotic signals are received. Then the following caspase activation cascades can activate downstream effector caspases such as caspase-3 or caspase-7 trigger the apoptotic execution phase (Budihardjo *et al.*, 1999; Green, 2000).

To date, anti-apoptotic genes such as *bcl*-2, *ced*-9, cowpox virus *crm*A, baculovirus inhibitor of apoptosis (*iap*), and *p35*, when overexpressed, prevent apoptosis induced by a variety of apoptotic agents in different systems (Vucic *et al.*, 1997). The *bcl*-2 family includes those that promote cell survival by inhibiting adaptors needed for activation of the caspases, whereas other members, such as Bax and Bak, of this family promote apoptosis (Reed, 1997; Adams and Cory, 1998). Membrane-bound localization of *bcl*-2 (mainly in mitochondria) helps to support the anti-apoptotic

property of this protein as a free radical scavenger and/or its ability to interact with other proteins such as cytochorome *c* involved in apoptotic induction (Kharbanda *et al.*, 1997; Green and Reed, 1998; Rossé *et al.*, 1998).

Anti-apoptotic *P35* protein is a potent inhibitor of the caspases, which accounts for its broad anti-apoptotic activity (Bump *et al.*, 1995; Xue and Horvitz, 1995; Bertin *et al.*, 1996). During infection of *Spodoptera frugiperda* SF21 cells with the prototype baculovirus *Autographa californica* nuclear polyhedrosis virus (Ac*M*NPV), viral-synthesized *P35* is cleaved by and inhibits a novel insect caspase (*S. frugiperda* caspase) (Bertin *et al.*, 1996). Stoichiometric interaction of active caspase with *P35* protein blocks protease activity by a mechanism that requires *P35* cleavage at residue Asp^{87} (Bump *et al.*, 1995; Xue and Horvitz, 1995; Bertin *et al.*, 1996). In addition to its ability to protect baculovirus-infected insect cells from apoptosis, ectopic expression of *p35* blocks apoptosis involving diverse death signals, e.g., developmental death, growth factor withdrawal, or DNA damage, in many different cell types and species (Villa *et al.*, 1997).

To date, an attachment-dependent cell line, *Trichoplusia ni* BTI Tn 5B1-4 cell has been shown to be superior to Sf9 or Sf21 lepidopteran cell lines for expression of both cytoplasmic and secreted glycosylated proteins (Wickham *et al.*, 1992; Davis *et al.*, 1993; Wickham and Nemerow, 1993; Lee *et al.*, 2001). Stably transformed Tn 5B1-4 cells have also been used for the expression of useful proteins as an alternative to the baculovirus expression system. And optimization of medium composition and bioreactor culture system for Tn 5B1-4 cells have been also described (Chung *et al.*, 1993; Wickham and Nemerow, 1993; Dee *et al.*, 1997). However, the suppression of apoptosis in the suspension culture of *Trichoplusia ni* BTI Tn 5B1-4 (Tn 5B1-4) in spinner flasks has not been investigated. Therefore, an attempt to maintain high-cell density culture was made by delaying onset of apoptosis during the spinner flask culture of Tn 5B1-4 cells. If the apoptosis of Tn 5B1-4 cells in the spinner flask culture can be delayed by overexpression of anti-apoptotic genes, this will be very useful for biotechnological applications. For this, We have established transformed Tn 5B1-4 cells using anti-apoptotic genes, *bcl*-2 and baculovirus *p35*, and analyzed for the function of those genes in spinner flask culture of stably transformed Tn 5B1-4 cells.

Materials and Methods

Cell line, plasmids and enzymes

Trichoplusia ni BTI Tn 5B1-4 (Tn 5B1-4) cells were grown at 27°C in Nunc (T-25; Roskilde, Denmark) flasks in Sf900II-SFM (serumfree medium; Gibco BRL, Grand Island, NY, USA) medium. The Tn 5B1-4 cells were a gift from Dr. Granados at Boyce Thompson Institute for Plant Research (Ithaca, N.Y., USA). The plasmid pIZT/V5-His (3.3 kb; Invitrogen, Carlsbad, CA, USA) contains an OpIE2 promoter, a V5 epitope tag, a polyhistidine region, and a zeocin resistance gene under the control of the EM7 promoter. *E. coli* DH5α was used as the primary host for constructing and propagating plasmids. *E. coli* cells were routinely grown and maintained in LB medium (1% tryptone, 0.5% yeast extract, and 0.5% NaCl, pH 7.3) containing 50 μg zeocin/ml (Invitrogen) with agitation at 37°C. We used DNA restriction enzymes from Promega (Madison, WI, USA) or Takara (Shiga, Japan) according to the manufacturers instructions.

Construction of expression plasmids containing anti-apoptotic genes

Bcl-2 gene was derived from pB4 (ATCC 79804). Plasmid pIZT/*bcl*-2/V5-His (Fig. 1) was constructed by inserting a *EcoR*I fragment of pB4 to pIZT/V5-His (Invitrogen). Also, baculovirus *p35* sequence was amplified from *Autographa californica* nucleopolyhedrosis virus (strain L-1) DNA by PCR using oligonucleotide primers. The sense primer was 5′-GGTACCATGTGTGTAATTTTTC-3′ and the antisense primer was 5′-GCGGCCGCCTT-

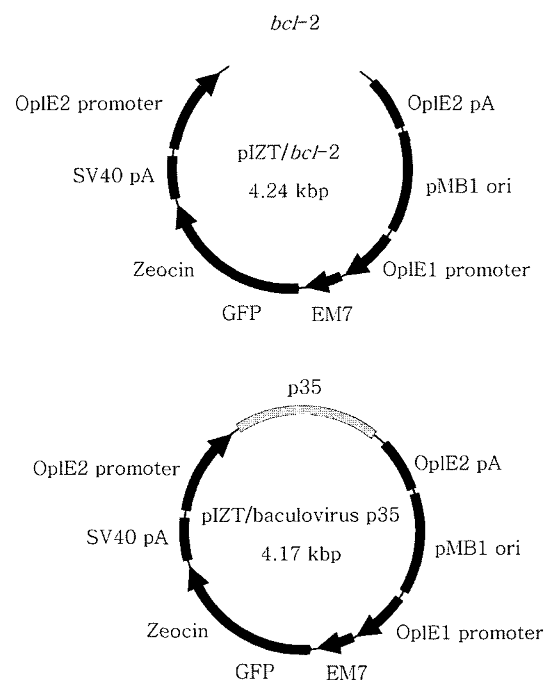

Fig. 1. Construction of anti-apoptotic genes expression plasmids.

TAATTGT G-3′. The amplified baculovirus *p35* sequence was then inserted into the pGEM-T vector (Promega) to yield pGEM-T/*p35*, and confirmed by DNA sequencing. PCR steps were performed in a Thermal Cycler (PE Biosystems, Foster City, CA, USA) using PCR mix (Takara) in a 50 μl volume. pIZT/*p35*/V5-His (Fig. 1) was constructed by inserting a *Kpn*I-*Not*I fragment of pGEM-T/*p35* between the *Kpn*I and *Not*I sites of pIZT/V5-His. The proper orientation and reading frame of the genes inserted in the recombinant plasmids of pIZT/*bcl*-2/V5-His and pIZT/*p35*/V5-His were confirmed by both restriction enzyme mapping and DNA sequencing.

Stable transformation and single clonal selection

Exponentially growing Tn 5B1-4 cells were transfected with plasmids pIZT/V5-His, pIZT/*bcl*-2/V5-His and pIZT/*p35*/V5-His using the lipofectin method. To prepare the transfection medium, plasmid DNA and lipofectin reagent (Gibco BRL) were separately diluted with Sf900II-SFM, and then mixed together in a ratio of 1 : 5. The transfection medium was incubated at room temperature for 15 min and transferred into 6-well plates preseeded 2 hrs earlier with Tn 5B1-4 cells in Sf900II-SFM. After 24 hrs of incubation, the medium was changed to remove the lipofectin and the cells were incubated for 4 more days in Sf900II-SFM without drug selection. Then, the medium was changed to selective Sf900II-SFM containing 400 μg zeocin/ml. The selective medium was replaced every 4 days. Stably transformed polyclonal cell populations were isolated after 4 weeks of selection with zeocin. Zeocin was maintained routinely with 200 μg/ml in the media at all times after selection. Single cell clones were selected by dilution method and conditioned medium was used to assist the growth of selected cell clones in 96 well plates.

Genomic DNA isolation and Southern blot hybridization

Total DNA was prepared by direct lysis of Tn 5B1-4 cell clones in 30 mM Tris (pH 8.0), 50 mM EDTA, 0.5% SDS and 150 mM NaCl, and treated for 5 hrs at 65°C with proteinase K (200 μg/ml), respectively. The solution was extracted with phenol chloroform and the high-molecular-weight DNA was collected with a sterile tip after isopropanol precipitation. The DNA was resuspended into a TE buffer (pH 8.0) and treated with Rnase A (20 mg/ml) for 1 hr. For genomic southern analysis, 15 μg of DNA were digested with the restriction enzymes for 24 hrs, subjected to electrophoresis on 0.8% agarose gel, transferred onto nylon membrane and hybridized to radiolabeled probes, respectively. Also, the equivalent DNA was indirectly estimated by haploid genome size of *lepidopteran Bombyx mori* (4.74×10^8 bp).

Apoptotic induction of cell clones in spinner flask culture

Tn 5B1-4 cell clones cultured for 3 – 4 days were washed with fresh medium. After cell counting, suspension cultures of Tn 5B1-4 cell clones were performed in spinner flask (Bellco, 100 ml vol.) at an initial density of 1×10^6 cell/ml with working volume of total 50 ml. The suspension medium contained 25 μg dextran sulfate/ml, to prevent cell clones from aggregating (Dee *et al.*, 1997), and 50 μg zeocin/ml. The operations of spinner flasks were performed at 100, 200 and 300 rpm for 8, 7 and 6 days, respectively. And then, cell number and viability were assessed by the trypan blue exclusion assay using a hemacytometer. Fragmentation of DNA was detected by agarose gel electrophoresis and morphological change of apoptotic cells was identified by inverted microscope (OLYMPUS, IX70, Japan).

SDS-PAGE and Western blot analysis

Protein samples were analyzed by SDS-PAGE according to the Laemmlis method (Laemmli, 1970). The electrophoresed proteins on the gel were transferred onto nitrocellulose, incubated with living color peptide antibody (Clontech, 1 : 100 v/v), mouse anti-*Bcl*-2 monoclonal antibody (ZYNED, 1 : 200 v/v) and mouse anti-V5 polyclonal antibody (Invitrogen, 1 : 1000 v/v), respectively. And then, these membranes were probed with anti-rabbit IgG alkaline phosphatase conjugate (Sigma, 1 : 1000 v/v) and anti-mouse IgG alkaline phosphatase conjugate (Sigma, 1 : 1000 v/v). After washing, BCIP/NBT solution (Amresco) was added and the reaction was quenched with distilled water.

Results and Discussion

Establishment of stably transformed single cell clones

We have attempted to study the function of anti-apoptotic genes using polyclonal Hi5 cells. But the results from polyclonal cell lines made us difficult to identify the function of anti-apoptotic genes due to the lack of reproducibility. So we have selected single cell clones expressing anti-apoptotic genes using dilution methods. Expression of anti-apoptotic proteins from selected cell clones were identified by western blot analysis. Hi5/pIZT control cell clones isolated were a total of 34 clones and only 7 clones (clone number 3, 15, 17, 20, 25, 30 and 31) expressing GFP were identified. Also, Hi5/pIZT/*bcl*-2 clones isolated were 5 clones (clone number 10, 15, 20, 24 and 46) expressing *Bcl*-2 among total 51 clones. Finally, Hi5/pIZT/*p35* clones isolated were 7 clones (clone number 4, 9, 12, 13, 18, 23 and 30) expressing baculovirus *P35*

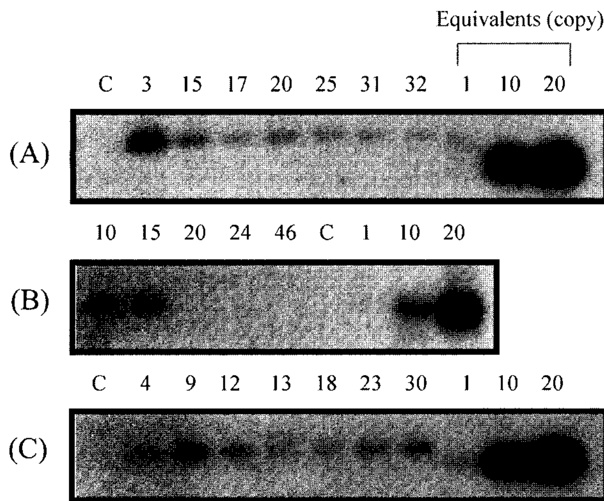

Fig. 2. Genomic Southern blot hybridization of stably transformed Hi5 cell clones. (A), (B) and (C) indicate Hi5/pIZT(3) control clone, Hi5/*bcl*-2(15) clone and Hi5/*p35*(23) clone, respectively. C is genomic DNA of non-transfected cells, equivalents 1, 10, 20 are calculated with haploid genome size of lepidopteran *Bombyx mori*.

among total 35 clones (data not shown). These results show that only 6 of 40 clones were expressing anti-apoptotic genes. The copy number of selected cell clones was determined by Southern blot analysis. The clones expressing anti-apoptotic proteins described above were confirmed as the ones to sustain about 1 – 15 copies of integrated anti-apoptotic gene. For later research, we have decided to use Hi5/pIZT(3), Hi5/pIZT/*bcl*-2(15) and Hi5/pIZT/*p35* (23) clones that sustain about 6 – 10 copies of integrated anti-apoptotic gene (Fig. 2). Also, the expression of anti-apoptotic protein from all cell clones was confirmed by Western blot analysis. The expression of fusion proteins of zeocin resistance gene and gfp gene was detected by living colors peptide antibody in cell extracts of Hi5/pIZT(3) clone. The expression of recombinant *BCL*-2 and *P35* proteins was also detected in Hi5/pIZT/*bcl*-2 (15) and Hi5/pIZT/*p35* (23) clone, respectively (Fig. 3).

Suspension culture of Tn 5B1-4 clones at 100 rpm

In a preliminary study, we have found out induction of apoptosis in suspension cultures of Hi5 cells using spinner flasks (data not shown). To investigate the effects of *Bcl*-2 and baculovirus *P35*, selected cell clones were cultured for eight days in spinner flasks at 100 rpm (Fig. 4). The cell growth of Hi5/pIZT/*bcl*-2(15) clone was much higher than other two clones and the maximum cell density of this clone was 6.0×10^6 cells/ml at day three after the incubation. Also, the maximum cell densities of Hi5/

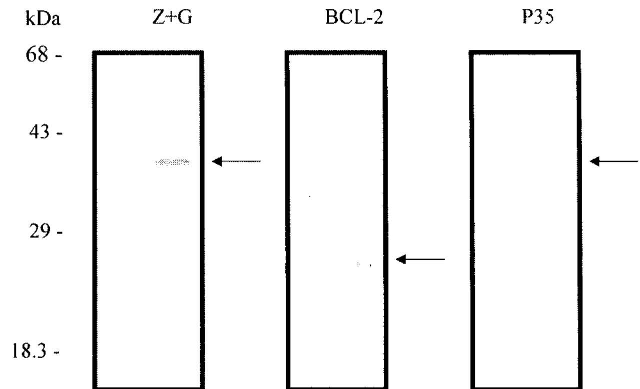

Fig. 3. Western blot analysis of stably transformed Hi5 cell clones. The arrow indicate the recombinant Zeocin^R + GFP fusion protein, *Bcl*-2 and baculovirus *P35* protein, respectively.

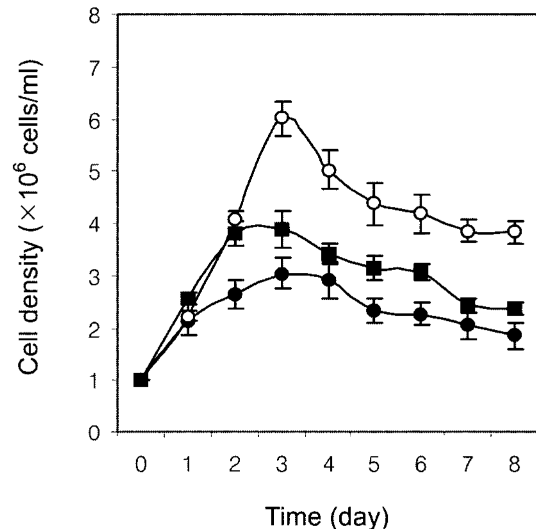

Fig. 4. Time-course changes of cell growth in spinner flask culture at 100 rpm. The cell concentrations are plotted against the incubation time when Hi5 cell clones are cultivated in spinner flasks at 100 rpm. Hi5/pIZT(3) clone (closed circle), Hi5/*bcl*-2(15) clone (open circle), Hi5/*p35*(23) clone (square).

pIZT(3) clone and Hi5/pIZT/*p35*(23) clone were 3.0×10^6 cells/ml and 3.9×10^6 cells/ml at day three after the incubation, respectively. Fig. 7A shows the electrophoresis results from those cultures of three different clones at day seven after incubation. The patterns of genomic DNA fragmentations indicating apoptotic induction were the similar in all clones. Since direct comparison based on the fragmentation of genomic DNAs was not feasible, we examined the induced apoptosis during the culture by microscopic observation of apoptotic bodies (Fig. 8). Cellular morphologies of Hi5/pIZT/*bcl*-2(15) and Hi5/pIZT/*p35* (23) clones were not nearly changed at day eight after the incubation. However, a little apoptotic bodies were

observed in the cells of Hi5/pIZT(3) clone. This result indicates that apoptosis can be induced in suspension culture of Hi5/pIZT(3) clone (control) at low agitation speed. The culture condition such as the agitation speed of 100 rpm would not provide the high shear stress to Tn 5B1-4 cells (Dee *et al.*, 1997). If the shear stress is not the main cause of the apoptosis at 100 rpm, other factor such as nutrient-related insult may play an important role in spinner flask culture. It has been known that *Trichoplusia ni* BTI Tn 5B1-4 (Hi5) cells are clumped in suspension culture (McKenna and Granados, 1994), however, clumping can be partly solved at higher agitation speed of 160 rpm by using dextran sulfate in culture medium (Dee *et al.*, 1997). Despite the presence of dextran sulfate, agitation speed of 100 rpm could induce Tn 5B1-4 cells to be clumped. The clumping of cells might induce apoptosis to Tn 5B1-4 cells due to nutrient-related insult. It has been reported that nutrient insult can be one of the factors for inducing apoptosis in batch and fed-batch cultures of hybridomas, Chinese hamster ovary (CHO), NSO, and Sf-9 cells (Vomastek and Franek, 1993; Mercille and Massie, 1994; Perreault and Lemieux, 1994; Singh *et al.*, 1994; Simpson *et al.*, 1998; Tintü *et al.*, 2002). As shown in figure 4, *Bcl-2* is provide to be more effective than *P35* in delaying onset of apoptosis in spinner flask culture at 100 rpm. Similar results on the function *Bcl-2* as well as baculovirus *P35* (Lin *et al.*, 2001) regarding nutrient insult were reported in hybridomas, myelomas, baby hamster kidney (BHK), CHO, and COS cells (Itoh *et al.*, 1995; Singh *et al.*, 1996; Mercille *et al.*, 1999; Mastrangelo *et al.*, 2000; Vives *et al.*, 2003).

Suspension culture of Tn 5B1-4 clones at 200 rpm

Likewise the results shown in suspension culture at 100 rpm, the cell growth of Hi5/pIZT/*bcl*-2(15) clone was better than other two clones at 200 rpm. The maximum cell density of this clone was 6.2×10^6 cells/ml at day three of the incubation (Fig. 5). Also, the maximum cell densities of Hi5/pIZT(3) clone and Hi5/pIZT/*p35*(23) clone were 5.1×10^6 cells/ml and 5.0×10^6 cells/ml, respectively. Hi5/pIZT(3) clone and Hi5/pIZT/*p35*(23) clone grew in a similar way from day zero to day three. Viable cell densities of Hi5/pIZT(3) were suddenly reduced after three days of incubation, whereas those of Hi5/pIZT/*p35*(23) clone maintained the same for seven days of incubation. Especially, cell growth of Hi5/pIZT/*bcl*-2(15) clone was much reduced than that of Hi5/pIZT/*p35*(23) clone after three days of incubation, forming rapid cell-death phase. Also, electrophoresis results of their genomic DNA from all clones indicate that the reduction of cell densities was related to apoptosis induction in Hi5 cells (Fig. 7B). Hi5/pIZT/*p35*(23) clone did not show any genomic DNA laddering, whereas Hi5/pIZT(3) clone showed more distinct than Hi5/pIZT/*bcl*-2(15) clone. This laddering phenomenon was also observed in Hi5/pIZT/*bcl*-2(15) clones. These results were reconfirmed by comparing morphological changes of all clones after seven days of incubation in the spinner flasks (Fig. 9). Hi5/pIZT/*p35*(23) clone maintained nearly the same cellular morphology with control Hi5 cells incubated for 4 days in T-25 flasks. On the other hand, Hi5/pIZT(3) clones have shown typical characteristics of apoptotic cells which result in fragmentation into apoptotic bodies. A little apoptotic bodies were observed in Hi5/pIZT/*bcl*-2(15) clone.

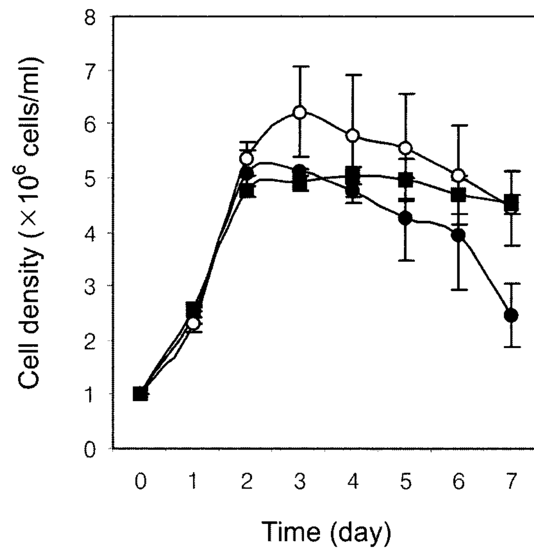

Fig. 5. Time-course changes of cell growth in spinner flask culture at 200 rpm. The cell concentrations are plotted against the incubation time when Hi5 cell clones are cultivated in spinner flasks at 200 rpm. Hi5/pIZT(3) clone (closed circle), Hi5/*bcl*-2(15) clone (open circle), Hi5/*p35*(23) clone (square).

Programmed cell death in the spinner flask culture at 200 rpm can be induced by the cultural environment such as nutrient insult, accumulation of waste products, or shear stress (Singh *et al.*, 1994; Itoh *et al.*, 1995; Singh *et al.*, 1996; Mercille *et al.*, 1999; Perani *et al.*, 1998). Overexpression of *bcl*-2 has been shown to limit or completely prevent apoptosis in a variety of cell types in response to a number of insults commonly encountered during normal bioreactor operation, including nutrient insult, accumulation of toxins, oxygen deprivation and hydrodynamic stress (Nunez *et al.*, 1990; Reed, 1994; Singh *et al.*, 1996; Shimizu *et al.*, 1996). In case of Hi5/pIZT/*bcl*-2 clone, although the clone is expressing anti-apoptotic *bcl*-2 proteins, unlike Hi5/pIZT/*p35*(23) clone, apoptosis was induced a little during the spinner flask culture. Our result shows that anti-apoptotic gene, *bcl*-2 is proven to be more effective than baculovirus *p35* at an agitation speed of 200 rpm.

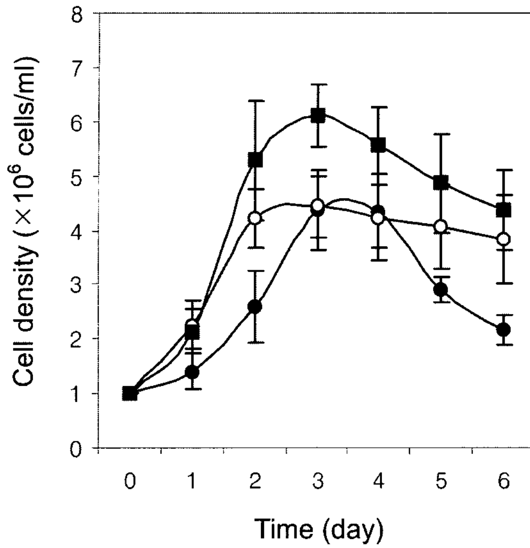

Fig. 6. Time-course changes of cell growth in spinner flask culture at 300 rpm. The cell concentrations are plotted against the incubation time when Hi5 cell clones are cultivated in spinner flasks at 300 rpm. Hi5/pIZT(3) clone (closed circle), Hi5/*bcl*-2(15) clone (open circle), Hi5/*p35*(23) clone (square).

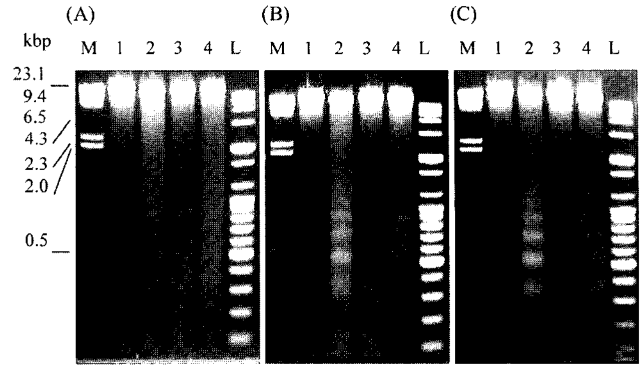

Fig. 7. Intranucleosomal fragmentations in spinner flask culture at 100 rpm (A), 200 rpm (B) and 300 rpm (C). (A), (B) and (C) are results of genomic DNA electrophoresis at day seven, six and five, respectively. Lanes (1), (2), (3), and (4) indicate genomic DNA of non-transfected cell, Hi5/pIZT(3) clone, Hi5/*bcl*-2(15) clone, and Hi5/*p35*(23) clone, respectively.

Suspension culture of Tn 5B1-4 clones at 300 rpm

In contrast with the results of suspension culture at 100 and 200 rpm, the cell growth rate of Hi5/pIZT/*p35*(23) clone was higher than other two clones. Its maximum cell density was 6.1×10^6 cells/ml at day three after the incubation. The maximum cell densities of Hi5/pIZT(3) and Hi5/pIZT/*bcl*-2(15) clones were 4.3×10^6 cells/ml and 4.4×10^6 cells/ml, respectively (Fig. 6). The induced phenomena in Hi5/pIZT/*p35*(23) clone at this condition were similar to those of Hi5/pIZT/*bcl*-2(15) clones at 200 rpm. At this condition the higher growth rate was observed but

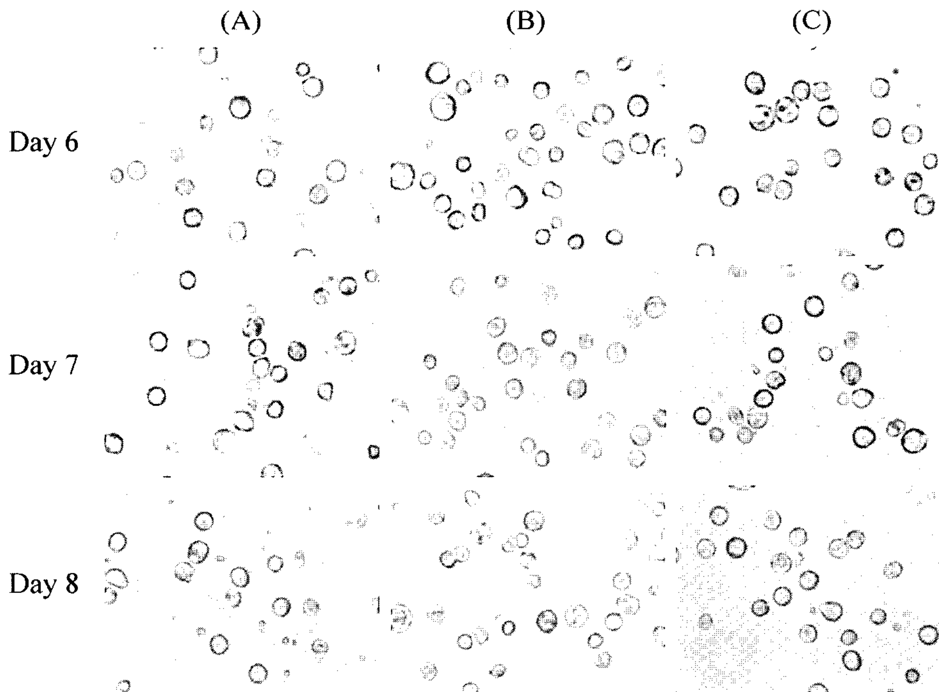

Fig. 8. Morphological changes of Hi5 clones in spinner flask culture at 100 rpm. (A), (B) and (C) indicate Hi5/pIZT(3) control clone, Hi5/pIZT/*bcl*-2(15) clone and Hi5/*p35*(23) clone, respectively. Above pictures show time-course results from day six to day eight.

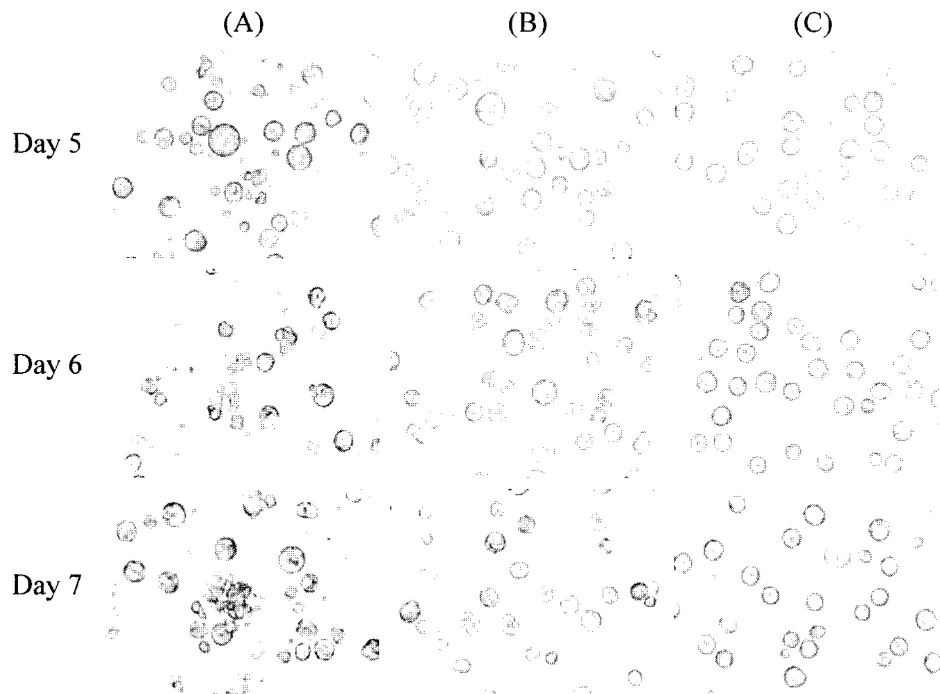

Fig. 9. Morphological changes of Hi5 clones in spinner flask culture at 200 rpm. Labels (A), (B) and (C) are equal to Fig. 8. Above pictures show time-course results from day five to day seven.

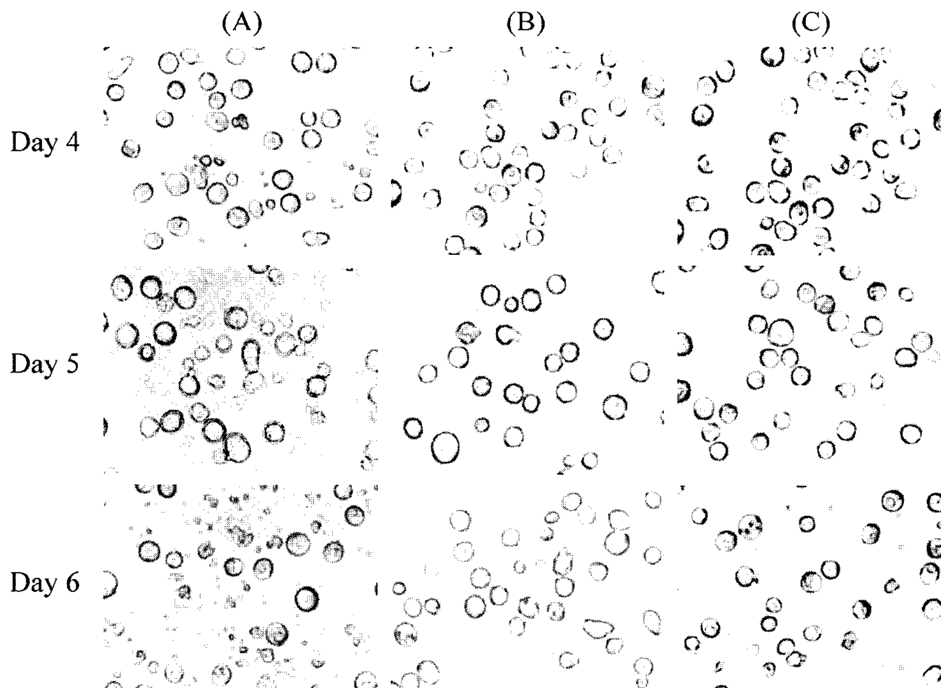

Fig. 10. Morphological changes of Hi5 clones in spinner flask culture at 300 rpm. Labels (A), (B) and (C) are equal to Fig. 8. Above pictures show time-course results from day four to day six.

apoptosis was also induced a little at six days of incubation. The Hi5/pIZT/*p35*(23) clone showed the similar results in the pattern of genomic DNA laddering (Fig. 7C) and in the microscopic observation of apoptotic bodies (Fig. 10). As shown in Figs. 4, 5 and 6, the higher agitation speed is increased, the more apoptotic bodies are

induced in Hi5/pIZT(3) clone. This indicates that the shear stress also can be a major factor inducing apoptosis in insect cell culture as well as mammalian cell culture (Singh *et al.*, 1994). At high stress levels, cells die by necrosis, largely because they have no time to respond to the stimulus and die instantly. Examples include high levels of toxins, sharp changes in pH and high agitation rate. At intermidiate levels of cell stress, the cell is injured but not killed and, as a result, has time to activate its own death program. Thus, the cell dies in a controlled way, by apoptosis, and actually participates in its own demise (Singh *et al.*, 1994; Cotter and Al-Rubeai, 1995). Although agitation speed of 300 rpm is enough high to induce necrosis, phenomena induced in this condition, genomic DNA laddering and fragmentation into apoptotic bodies of cells, were similar to the apoptosis phenomena. Also, if it was due to necrosis, cellular debris could have detected at early stage of the suspension culture by microscopic observation; however, we could not observe any cell debris (data not shown). Overexpression *Bcl*-2 has been shown to prevent apoptosis induced in response to hydrodynamic stresses during bioreactor operation (Cotter and Al-Rubeai 1995; Singh *et al.*, 1996). However, anti-apoptotic abilities of baculovirus *P35* at high hydrodynamic stress have not been described yet.

It is possible that the higher agitation speed can be induced the higher oxidative stress. *Bcl*-2 as well as baculovirus *P35* function in an antioxidant pathway to prevent apoptosis (Hockenbery *et al.*, 1993; Sah *et al.*, 1999). In particular, *Bcl*-2 can function through protein-protein interactions (Hockenbery *et al.*, 1993) while baculovirus *P35* is able to quench the *in vitro* and *in vivo* generated superoxide radicals at an upstream step of the apoptotic signaling cascade (Sah *et al.*, 1999). In this study, we first report that the cell growth rate of cell clone expressing baculovirus *P35* was better than the clones expressing *bcl*-2 at a higher agitation speed, such as 300 rpm. The reason for that is not clearly known at this time.

We have established Hi5 cell clones expressing anti-apoptotic proteins, *Bcl*-2 and baculovirus *P35*, for the delay of onset of apoptosis in a spinner flask culture. Our results show that Hi5 cell clones expressing *Bcl*-2 was much effective than a control cell clone with respect to cell growth and suppression of induced apoptosis at an agitation speed of 100 and 200 rpm. On the other hands, Hi5 cell clones expressing baculovirus *P35* was much effective at high agitation speed such as 300 rpm. To date, this is first report that anti-apoptotic genes, *Bcl*-2 and baculovirus *P35*, can play a distinct role dependent upon the agitation speed in the spinner flask culture. Our findings demonstrate that transformed Hi5 cell lines harboring anti-apoptotic genes delay the onset of apoptosis in spinner flask culture more than 72 hrs, compared to the control. It is also possible that the productivity of the spinner flask culture can be improved by either combination of recombinant baculovirus vector system/insect cells expressing anti-apoptotic genes, or simultaneous expression of anti-apoptotic genes and useful genes in Tn 5B1-4 cells.

References

Adams, J. M. and S. Cory (1998) The *Bcl*-2 protein family: arbiters of cell survival. *Science* **28**, 1322-1326.

Alnemri, E. S., N. M. Robertson, T. F. Fernandes, C. M. Croce and G. Litwack (1992) Overexpressed full-length human BCL2 extends the survival of baculovirus-infected Sf9 insect cells. *Proc. Natl. Acad. Sci. USA* **89**, 7295-7299.

Bertin, J., S. M. Mendrysa, D. J. LaCount, S. Gaur, J. F. Krebs, R. C. Armstrong, K. J. Tomaselli and P. D. Friesen (1996) Apoptotic suppression by baculovirus *P35* involves cleavage by and inhibition of a virus-induced CED-3/ICE-like protease. *J. Virol.* **70**, 6251-6259.

Budihardjo, I., H. Oliver, M. Lutter, X. Luo and X. D. Wang (1999) Biochemical pathways of caspase activation during apoptosis. *Annu. Rev. Cell Dev. Biol.* **15**, 269-290.

Bump, N. J., M. Hackett, M. Hugunin, S. Seshagiri, K. Brady, P. Chen, C. Ferenz, S. Franklin, T. Ghayur, P. Li, P. Licari, J. Mankovich, L. Shi, A. H. Greenberg, L. K. Miller and W. W. Wong (1995) Inhibition of ICE family proteases by baculovirus antiapoptotic protein *p35*. *Science* **269**, 1885-1888.

Chung, I. S., R. A. Taticek and M. I. Shuler (1993) Production of human alkaline phosphatase, a secreted glycosylated protein, from a baculovirus expression system and the attachment-dependent cell line *Trichoplusia ni* BTI Tn 5B1-4 using a split-flow air-lift bioreactor. *Biotechnol. Prog.* **9**, 675-678.

Cotter, T. G. and M. Al-Rubeai (1995) Cell death (apoptosis) in cell culture systems. *Trends Biotechnol.* **13**, 150-155.

Dee, K. U., M. L. Shuler and H. A. Wood (1997) Inducing single-cell suspension of BTI-TN5B1-4 insect cells: I. The use of sulfated polyanions to prevention cell aggregation and enhance recombinant protein production. *Biotechnol. Bioeng.* **54**, 191-205.

Green, D. R. and J. C. Reed (1998) Mitochondria and apoptosis. *Science* **281**, 1309-1312.

Green, D. R. (2000) Apoptotic pathway: paper wraps stone blunts scissors. *Cell* **102**, 1-4.

Hockenbery, D. M., Z. N. Oltvai, X. M. Yin, C. L. Milliman and S. J. Korsmeyer (1993) *Bcl*-2 functions in an antioxidant pathway to prevent apoptosis. *Cell* **75**, 241-251.

Itoh, Y., H. Ueda and E. Suzuki (1995) Overexpression of *bcl*-2, apoptosis suppressing gene: Prolonged viable culture period of hybridoma and enhanced antibody production. *Biotechnol. Bioeng.* **48**, 118-122.

Kharbanda, S., P. Pandey, L. Schofield, S. Israels, R. Roncin-

ske, K. Yoshida, A. Bharti, Z. M. Yuan, S. Saxena, R. Weichselbaum, C. Nalin and D. Kufe (1997) Role for Bcl-xL as an inhibitor of cytosolic cytochrome C accumulation in DNA damage-induced apoptosis. *Proc. Natl. Acad. Sci. USA* **94**, 6939-6942.

Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of head of bacteriophage. *Nature* **227**, 680-685.

Lee, J. M., K. H. Chang, J. H. Park, Y. H. Lee and I. S. Chung (2001) Production of recombinant endostatin from stably transformed *Trichoplusia ni* BTI Tn 5B1-4 cells. *Biotechnol. Lett.* **23**, 1931-1936.

Lin, G., G. Li, R. R. Granados and G. W. Blissard (2001) Stable cell lines expressing baculovirus *P35*: resistance to apoptosis and nutrient stress, and increased glycosylation secretion. *In vitro Cell. Dev. Biol. Anim.* **37**, 293-302.

Mastrangelo, A. J., J. M. Hardwick, B. Zou and M. J. Betenbaugh (2000) Part II. Overexpression of *bcl*-2 family members enhances survival of mammalian cells in response to various culture insults. *Biotechnol. Bioeng.* **67**, 555-565.

Mercille, S. and B. Massie (1994) Induction of apoptosis in nutrient deprived cultures of hybridoma and myeloma cells. *Biotechnol. Bioeng.* **44**, 1140-1154.

Mercille, S., P. Jolicoeur, C. Gervais, D. Paquette, D. D. Mosser and B. Massie (1999) Dose-dependent reduction of apoptosis in nutrient-deprived cultures of NS/0 myeloma cells transfected with the E1B-19K adenoviral gene. *Biotechnol. Bioeng.* **63**, 516-528.

McKenna, K. A. and R. R. Granados (1994) Methods of adapting anchorage-dependent cell lines to suspension conditions. U. S. patent: 5,348,877.

Nunez, G., L. London, D. Hockenbery, M. Alexander, J. P. McKearn and S. J. Korsmeyer (1990) Deregulated *Bcl*-2 gene expression selectively prolongs survival of growth factor-deprived hemopoietic cell lines. *J. Immunol.* **144**, 3602-3610.

Perani, A., R. P. Singh, R. Chauhan and M. Al-Rubeani (1998) Variable functions of *bcl*-2 in mediating bioreactor stress-induced apoptosis in hybridoma cells. *Cytotechnology* **28**, 177-188.

Perreault, J. and R. Lemieux (1994) Essential role of optimal protein synthesis in preventing the apoptotic death of cultured B cell hybridomas. *Cytotechnology* **13**, 99-105.

Reed, J. C. (1994) *Bcl*-2 and the regulation of programmed cell death. *J. Cell Biol.* **124**, 1-6.

Reed, J. C. (1997) Double identity for proteins of the *Bcl*-2 family. *Nature* **19**, 773-776.

Rosse, T., R. Olivier, L. Monney, M. Rager, S. Conus, I. Fellay, B. Jansen and C. Borner (1998) *Bcl*-2 prolongs cell survival after Bax-induced release of cytochrome c. *Nature* **391**, 496-499.

Sah, N. K., T. K. Taneja, N. Pathak, R. Begum, M. Athar and S. E. Hasnain (1999) The baculovirus antiapoptotic *p35* gene also functions via an oxidant-dependent pathway. *Proc. Natl. Acad. Sci. USA* **96**, 4838-4843.

Shimizu, S., Y. Eguchi, W. Kamiike, Y. Itoh, J. Hasegawa, K. Yamabe, Y. Otsuki, H. Matsuda and Y. Tsujimoto (1996) Induction of apoptosis as well as necrosis by hypoxia and predominant prevention of apoptosis by *Bcl*-2 and Bcl-XL. *Cancer Res.* **56**, 2161-2166.

Simpson, N. H., R. P. Singh, A. Perani, C. Goldenzon and M. Al-Rubeai (1998) In hybridoma cultures, deprivation of any single amino acid leads to apoptotic death, which is suppressed by the expression of the *bcl*-2 gene. *Biotechnol. Bioeng.* **59**, 90-98.

Singh, R. P., M. Al-Rubeai, C. D. Gregory and A. N. Emery (1994) Cell death in boreactors: a role for apoptosis. *Biotechnol. Bioeng.* **44**, 720-726.

Singh, R. P., A. N. Emery and M. Al-Rubeai (1996) Enhancement of survivability of mammalian cells by over expression of the apoptosis suppressor gene *bcl*-2. *Biotechnol. Bioeng.* **52**, 166-175.

Singh, R. P., G. Finka, A. N. Emery and M. Al-Rubeai (1997) Apoptosis and its control in cell culture systems. *Cytotechnology* **23**, 87-93.

Tintó, A., C. Gabernet, J. Vives, E. Prats, J. J. Cairó, L. Cornudella and F. Gódia (2002) The protection of hybridoma cells from apoptosis by caspase inhibition allows culture recovery when exposed to non-inducing conditions. *J. Biotechnol.* **95**, 205-214.

Villa, P., S. H. Kaufmann and W. C. Earnshaw (1997) Caspases and caspase inhibitors. *Trends Biochem Sci.* **22**, 388-393.

Vives, J., S. Juanola, J. J. Cairü, E. Prats, L. Cornudella and F. Güdia (2003) Productive effect of viral homologues of *bcl*-2 on hybridoma cells under apoptosis-inducing conditions. *Biotechnol. Prog.* **19**, 84-89.

Vomastek, T. and F. Franek (1993) Kinetics of development of spontaneous apoptosis in B cell hybridoma cultures. *Immunol. Lett.* **35**, 19-24.

Vucic, D., S. Seshagiri and L. K. Miller (1997) Characterization of reaper- and FADD-induced apoptosis in a lepidopteran cell line. *Mol. Cell. Biol.* **17**, 667-676.

Wickham, T. J. and G. R. Nemerow (1993) Optimization of growth methods and recombinant protein production in BTI-Tn5B1-4 insect cells using the baculovirus expression system. *Biotechnol. Prog.* **9**, 25-30.

Wyllie, A. H., J. F. R. Kerr and A. R. Currie (1980) Cell death: the significance of apoptosis. *Int. Rev. Cytol.* **68**, 251-306.

Xue, D. and H. R. Horvitz (1995) Inhibition of the *Caenorhabditis elegans* cell-death protease CED-3 by a CED-3 cleavage site in baculovirus *p35* protein. *Nature* **377**, 248-251.