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Bcl-2 blocks apoptosis caused by pierisin-1, a guanine-specific ADP-ribosylating toxin from the cabbage butterfly

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Abstract

Pierisin-1, a 98-kDa protein that induces apoptosis in mammalian cell lines, is capable of being incorporated into cells where it ADP-ribosylates guanine residues in DNA. To investigate the apoptotic pathway induced by this unique protein, the *bcl-2* gene was transfected into HeLa cells. Cy2-fluorescent pierisin-1 was incorporated into the resultant cells expressing Bcl-2 protein and ADP-ribosylated dG was detected to almost the same extent as in parent cells. However, *bcl-2*-transfected HeLa cells did not display apoptotic morphological changes, PARP cleavage, and DNA fragmentation, indicating acquisition of resistance. In parent HeLa cells, activation of caspase-9 and release of cytochrome *c* were observed after 8 h treatment with 0.5 ng/ml pierisin-1. Caspase substrate assays revealed further cleavage of Ac-DEVD-pNA, Ac-VDVAD-pNA, and Ac-VEID-pNA, suggesting activation of caspase-2, -3, and -6 in pierisin-1-treated HeLa cells. The caspase-3 inhibitor, Ac-DEVD-CHO, was also found to inhibit apoptosis. In contrast, this caspase activation was not observed in *bcl-2*-transfected HeLa cells. Our results thus indicate that pierisin-1-induced apoptosis is mediated primarily via a mitochondrial pathway involving Bcl-2 and caspases.

Keywords: Apoptosis; Pierisin-1; HeLa cells; Caspases; Bcl-2 protein

We have previously reported that the cabbage butterfly *Pieris rapae* contains a protein, which is highly cytotoxic to human carcinoma cell lines [1]. This pierisin-1 is a 98-kDa protein [2] comprising 850 amino acids sharing regional sequence similarity with ADP-ribosylating toxins such as the A-subunit of cholera toxin [3]. As with other ADP-ribosylating toxins, pierisin-1 has an enzyme domain that exhibits ADP-ribosyltransferase activity and a receptor-binding domain that incorporates the protein into the cell by interaction with receptors on cell membranes. The most likely candidate for the pierisin-1 receptor is a glycolipid whose presence has been suggested to reflect sensitivity to pierisin-1 [4]. Moreover, the targets of ADP-ribosylation by pierisin-1 were recently revealed to be 2′-deoxyguanosine residues in DNA. The ADP-ribose moiety of NAD was transferred to N^2 of 2′-deoxyguanosine to yield N^2-(α-ADP-ribos-l-yl)-2′-deoxyguanosine and its β form. Furthermore, ADP-ribosylated dG was detected in DNA from pierisin-1-treated cells, in which apoptosis was readily induced [5].

Pierisin-1 exhibits potent cytotoxic effects against various types of mammalian cancer cell lines and human umbilical vein endothelial cells, with IC_{50} values ranging from 0.043 to 270 ng/ml, and induces typical apoptotic changes such as PARP cleavage and DNA fragmentation [4,6]. Of a number of lines tested, the cervical carcinoma cell line, HeLa, proved the most sensitive. It is plausible that formation of ADP-ribosylated dG in DNA is associated with induction of apoptosis by pierisin-1. However, the mechanisms of apoptosis remained to be clarified in detail.

Apoptosis is characterized by chromatin condensation and DNA fragmentation, and is mediated by the cysteine protease family called caspases [7,8]. Mitochondria are

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involved in a variety of key events, including the release of caspase activators, changes in electron transport, and loss of mitochondrial transmembrane potential, and participation of both pro- and anti-apoptotic Bcl-2 family proteins has been proposed [9,10].

Bcl-2 itself is known to exert anti-apoptotic effects [11,12], preventing the release of mitochondrial proteins such as cytochrome *c* [13,14] or apoptosis-inducing factor (AIF) [15] into the cytosol, by maintaining the mitochondrial membrane potential. Apoptosis signals caused by DNA damage or cytotoxic drug treatment as well as by Fas ligand or the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) can be prevented by Bcl-2 [16–18]. Moreover, some bacterial components such as diphtheria and Shiga-like toxins cause apoptosis, accompanied by caspase activation [19] and this is inhibited by Bcl-2 [20].

In the present study, we transfected the *bcl-2* gene into HeLa cells to investigate its effect on pierisin-1-induced apoptosis. Caspase activation and release of cytochrome *c* were also examined in parental and *bcl-2*-transfected cells, to cast light on the apoptosis pathway involved.

Materials and methods

Cell lines. Human cervical carcinoma HeLa cells were obtained from the RIKEN cell bank (Tsukuba, Japan) and the human *bcl-2* gene, inserted into expression vector pcDNA3, was provided by Dr. Chifumi Kitanaka, Biophysics Division, National Cancer Center Research Institute. HeLa cells transfected with *bcl-2* (HeLa/bcl-2) were constructed by lipofection using the Effectene Transfection Reagent (Qiagen, Hilden, Germany) and cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (GIBCO/BRL, Gaithersburg, MD) and 500 μg/ml G418. Cell cultures were maintained at 37 °C in an atmosphere of 95% air and where appropriate 5% CO_2.

Visualization of pierisin-1 by fluorescence-labeling. Pierisin-1 protein obtained from *P. rapae* [2] was fluorescence-labeled with the Fluoro-Link-Ab Cy2 Labeling Kit (Amersham Pharmacia Biotech, Little Chalfont, UK) using the method recommended by the manufacturer [4]. Binding and internalization of the labeled pierisin-1 into cultured cells were observed under a fluorescence microscope.

Analysis of ADP-ribosylated dG in DNA from pierisin-1-treated HeLa and HeLa/bcl-2 cells. Samples for detection of DNA adducts were obtained as follows. One ng/ml pierisin-1 was added to 5×10^6 cultured HeLa or HeLa/bcl-2 cells and incubated at 37 °C for the indicated times. These cells were then collected using a cell scraper and immediately extracted with phenol and chloroform/isoamyl alcohol (24:1 v/v). After enzymatic digestion of protein and RNA, DNA was re-extracted. DNA samples thus obtained were subjected to the ^{32}P-postlabeling method as described previously [5].

Analysis of apoptosis-inducing activity. Apoptosis-inducing activity of pierisin-1 was confirmed by morphological analysis of nuclei from cells stained with Hoechst 33342 under a fluorescence microscope. Analysis of DNA fragmentation was as described previously [6].

Colorimetric assay of caspase activity with pNA-labeled substrates. Caspase substrates were purchased from Calbiochem (San Diego, CA). Cultured HeLa and HeLa/bcl-2 cells were treated with 1 ng/ml pierisin-1. After 4, 8, 12, 18, and 24 h, cells were collected with a cell scraper, washed with PBS, mixed with lysis buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 0.1% CHAPS, 1 mM DTT, and 0.1 mM EDTA) for 10 min at 0 °C, centrifuged at 10,000*g* for 10 min at 4 °C, and subjected to caspase colorimetric assays. Twenty μl supernatant fluid containing 100 μg protein was added to 80 μl assay buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 0.1% CHAPS, 10 mM DTT, 0.1 mM EDTA, and 10% glycerol) containing 200 μM caspase substrates to make a total of 100 μl solution and incubated for 2 h at 37 °C. The absorbance was then monitored at 405 nm to determine the caspase activity.

Inhibition of caspase. Peptide inhibitor of caspase-3 (Ac-DEVD-CHO) was purchased from Calbiochem. HeLa cells were pre-treated with 1 mM caspase inhibitor for 1 h and then 0.2 ng/ml pierisin-1 was added to the inhibitor-containing medium. Inhibitory effects on pierisin-1-induced cytotoxicity were examined after 24 h under microscope.

Western blot analysis of cell fractions. Pierisin-1-treated HeLa cells (1×10^7) were collected with a cell scraper, washed in PBS, then fractionated with a standard procedure [21], separated by SDS–polyacrylamide gel electrophoresis, and blotted onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA) by semi-dry electrophoretic transfer. Cytosolic fractions were used for detection of caspase-9 and release of cytochrome *c* from mitochondria, and nuclear fractions were for detection of PARP cleavage. For these procedures, anti-caspase-9 antibodies from Santa Cruz Biotechnology (Santa Cruz, CA) and PARP and cytochrome *c* antibodies from BD PharMingen (San Diego, CA) were employed. Horseradish peroxidase-conjugated antirabbit or -mouse IgG (Amersham Pharmacia Biotech) was used as the secondary antibodies. Protein–antibody complexes were visualized with an ECL chemiluminescence kit (Amersham Pharmacia Biotech).

Results

Acquisition of resistance to pierisin-1-induced apoptosis in bcl-2-transfected HeLa cells

The *bcl-2*-transfected HeLa cells (HeLa/bcl-2) constructed by lipofection demonstrated stable elevated expression of Bcl-2 as confirmed by Western blot analysis (Fig. 1A). When fluorescence-labeled pierisin-1 was added to HeLa and HeLa/bcl-2 cells with incubation at 37 °C for 2 h, strong fluorescence was detected in both the HeLa and HeLa/bcl-2 cells after 2 h of treatment as shown in Fig. 1B. Assessment of levels of ADP-ribosylated dG adducts in cells treated with pierisin-1 using the ^{32}P-postlabeling method was accomplished after 4 or 12 h of incubation of HeLa and HeLa/bcl-2 cells with 1 ng/ml pierisin-1. In both HeLa and HeLa/bcl-2 cells, two distinct spots that were derived from α- and β-isoforms of ADP-ribosylated dG were detected (Fig. 1C). The relative adduct labeling of ADP-ribosylated dG was 1.5 per 10^5 nucleotides in HeLa and 1.2 per 10^5 nucleotides in HeLa/bcl-2 at 4 h. At 12 h, the relative adduct labeling was 10 and 14 per 10^5 in HeLa and HeLa/bcl-2 cells, respectively. These results suggested pierisin-1 to be internalized and to ADP-ribosylate DNA in both HeLa and HeLa/bcl-2 cells to almost the same extent.

We then examined pierisin-1-induced apoptosis in HeLa and HeLa/bcl-2 cells after addition of 0.5 ng/ml and incubation at 37 °C for 24 h. The HeLa cells displayed typical apoptotic features, such as chromatin condensation and nuclear fragmentation. However, no such changes were induced in HeLa/bcl-2 cells (Fig. 2A).

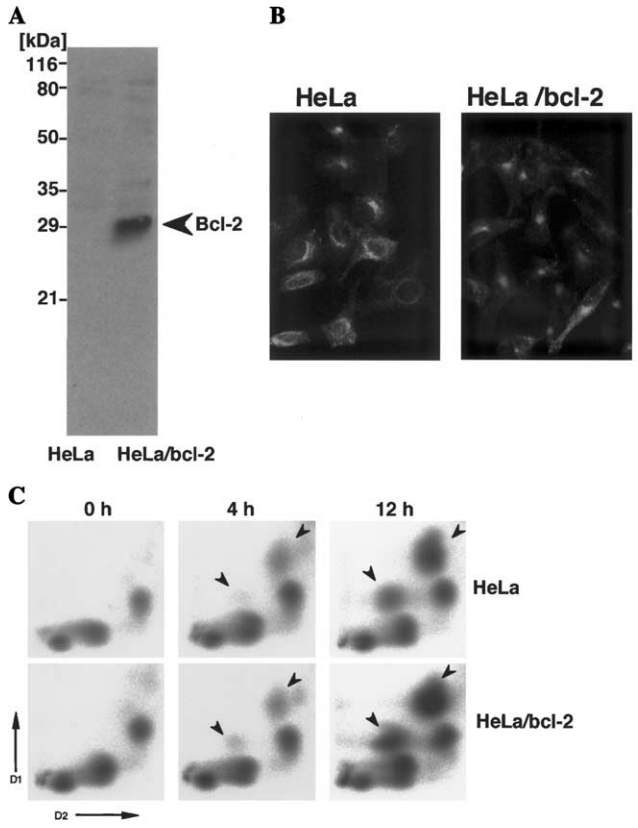

Fig. 1. Effects of Bcl-2 protein on incorporation of pierisin-1 and ADP-ribosylation of cellular DNA. (A) Detection of Bcl-2 protein. HeLa and *bcl-2*-transfected HeLa cells (HeLa/bcl-2) were collected, lysed with SDS, and subjected to Western blot analysis. The arrowhead indicates Bcl-2 protein. (B) Fluorescence micrographs of Cy2-pierisin-1-treated HeLa and HeLa/bcl-2 cells. Both cell lines were incubated with 2 μg/ml Cy2-pierisin-1 for 2 h at 37 °C. (C) Autoradiograms of DNA adducts in HeLa and HeLa/bcl-2 cells treated with 1 ng/ml pierisin-1 for 4 and 12 h. DNA adduct spots derived from pierisin-1 treatment are indicated by arrowheads.

DNA fragmentation was also observed in HeLa but not HeLa/bcl-2 cells after 12–24 h of treatment with pierisin-1 (data not shown). Finally, PARP cleavage was examined in the nuclear fractions of HeLa and HeLa/bcl-2 cells treated with pierisin-1. Cleaved PARP appeared in HeLa cells treated for 8 h with pierisin-1 and was persistently observed up to 24 h (Fig. 2B). However, only a small amount of cleaved PARP was observed in HeLa/bcl-2 cells, even after 24 h of treatment. These results indicated that the pierisin-1-induced apoptosis pathway was blocked by Bcl-2 protein.

Release of cytochrome c and activation of downstream caspases by pierisin-1

Bcl-2 has been reported to inhibit the mitochondria-related apoptosis pathway. Therefore, release of cytochrome *c* into the cytosol was examined in cells treated with 0.5 ng/ml of pierisin-1 by Western blot analysis. Cytochrome *c* was detected in the cytosolic fraction of HeLa cells at 8 h and the intensity of its band increased following treatment. In contrast, some amount of cytochrome *c* was detected in the cytosol of untreated HeLa/bcl-2 cells and its level was not significantly affected by pierisin-1 treatment (Fig. 3A).

Activation of caspase-9, which is considered to be a downstream mediator of the mitochondrial pathway, was also examined. Fig. 3B shows that the 46-kDa band, corresponding to pro-caspase-9, decreased in a time-dependent fashion from 8 h after pierisin-1 treatment in HeLa cells. Conversely, a 37-kDa band, considered to represent a degraded fragment of caspase-9, simultaneously increased. This degraded fragment was not observed in HeLa/bcl-2 cells.

To examine activation of other caspases, we used p-nitroaniline (pNA)-labeled caspase substrates and performed caspase colorimetric assays. Caspase-3 activity (Ac-DEVD-pNA), caspase-6 activity (Ac-VEID-pNA),

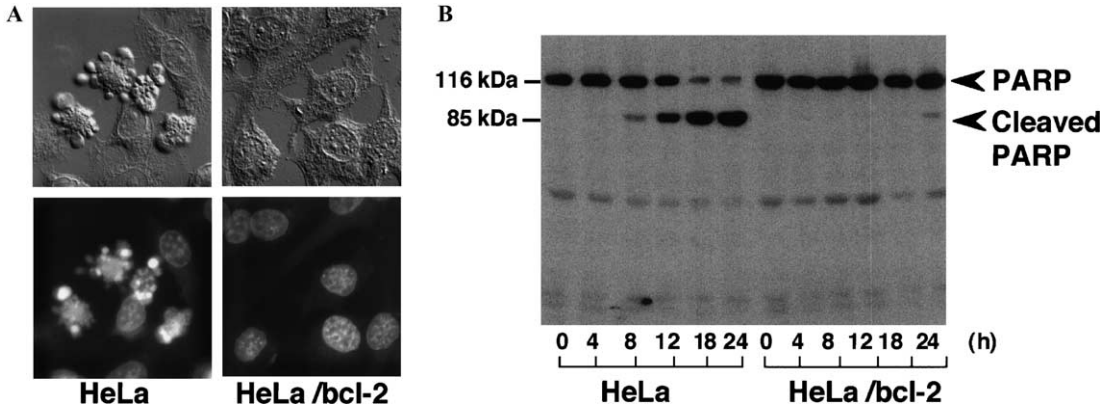

Fig. 2. Apoptotic changes in HeLa and HeLa/bcl-2 cells treated with pierisin-1. (A) Phase-contrast (upper part) and fluorescence (lower part) micrographs of HeLa and HeLa/bcl-2 cells treated with 0.5 ng/ml pierisin-1 for 24 h. Hoechst 33342 was used for nuclear staining of fixed cells. (B) PARP cleavage in HeLa and HeLa/bcl-2 cells treated with 0.5 ng/ml pierisin-1. Both cell lines were treated with pierisin-1 for 4, 8, 12, 18, or 24 h at 37 °C. Nuclear fractions were then subjected to Western blot analysis.

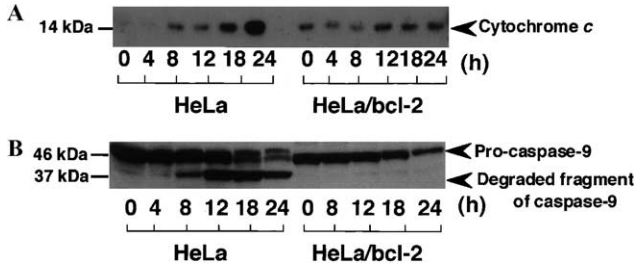

Fig. 3. Release of cytochrome *c* from mitochondria into cytosol and activation of caspase-9 during pierisin-1-mediated apoptosis. Detection of (A) cytosolic cytochrome *c* and (B) cleavage of caspase-9 in HeLa and HeLa/bcl-2 cells treated with pierisin-1. Both cell lines were treated with 0.5 ng/ml pierisin-1 for 4, 8, 12, 18, or 24 h at 37 °C. Cytosolic fractions were then subjected to Western blot analysis.

and caspase-2 activity (Ac-VDVAD-pNA) increased in a time-dependent manner by about 20-fold at 18 h after treatment with pierisin-1 (Fig. 4). No such remarkable change was observed in HeLa/bcl-2 cells. Caspase-8 activity and caspase-1, -4, and -5 activities were only weakly increased in HeLa cells (Fig. 4).

To confirm whether activation of the caspases contributed to apoptosis, an inhibitor of caspase-3 protease, Ac-DEVD-CHO, was used. As shown in Fig. 5, cell death caused by pierisin-1 was clearly inhibited when cells were pre-treated with Ac-DEVD-CHO.

Discussion

In the present study, we obtained clear evidence that the apoptosis induced by pierisin-1 is due to a mitochondrial pathway inhibited by Bcl-2. Resistance to pierisin-1-induced apoptotic morphological changes was evident in cells transfected with Bcl-2. Since Bcl-xL is also a member of the Bcl-2 subfamily protein and acts as an apoptosis-inhibitor, like Bcl-2 [22,23], its effects on pierisin-1-induced apoptosis were also preliminarily examined. Similar resistance was observed with mouse *bcl-xL*-transfected human gastric carcinoma TMK-1 cells in a separate experiment. These results suggest that the pierisin-1-induced apoptosis pathway is blocked by both anti-apoptotic Bcl-2-subfamily proteins.

Cy-2-pierisin-1 was found to be efficiently incorporated into both HeLa and HeLa/bcl-2 cells and, moreover, ADP-ribosylated dG adducts were detected at similar levels over the same time course. Thus, the inhibition was not due to any inability of the toxin to be incorporated into the cell or ADP-ribosylate target DNA.

Bcl-2 overexpression has been reported to inhibit apoptosis but not to alter overall survival after treatment with aphidicolin or UV-C radiation [24,25]. Our data also showed that prolonged incubation of HeLa/bcl-2 cells with pierisin-1 indued apparent growth inhibition and detachment of the cells without any typical apoptotic morphology, as with UV irradiation or diphtheria toxin treatment (data not shown). These results are not only consistent with the notion that pierisin-1 was incorporated and formed ADP-ribosylated dG in HeLa and HeLa/bcl-2 cells in the same way, but also suggest that the death signal which is upstream of Bcl-2 activity was already triggered by the treatment.

Cytosolic cytochrome *c* binds to Apaf-1, which in turn promotes activation of pro-caspase-9. Caspase-9 is known as the initiator caspase, which activates effector

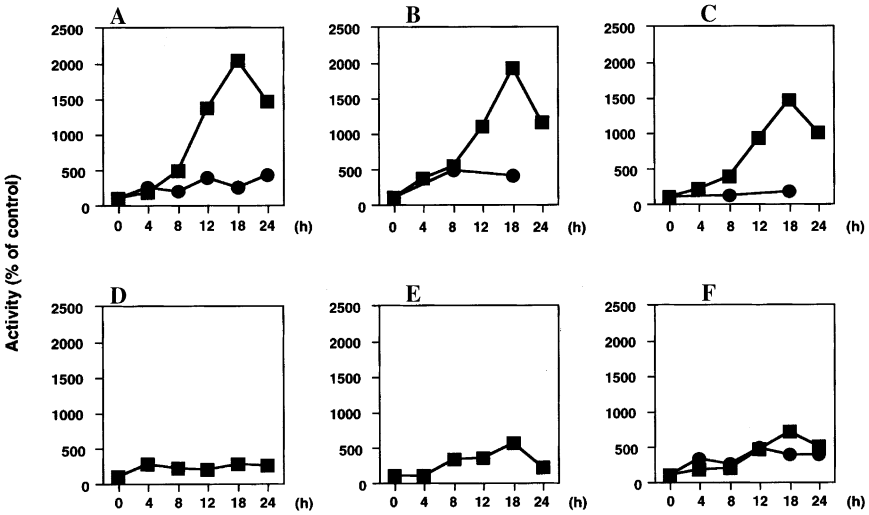

Fig. 4. Activation of caspases during pierisin-1-mediated apoptosis. HeLa (■) and HeLa/bcl-2 (●) cell lines were treated with 0.5 ng/ml pierisin-1 for 4, 8, 12, 18, or 24 h at 37 °C, lysed, and centrifuged. The supernatant was then added to 200 μM caspase substrate and subjected to caspase colorimetric assay. Protease activities of caspases were calculated by monitoring the absorbance at 405 nm. Data shown are percentages of the control value (0 h). (A) Caspase-3 activity (Ac-DEVD-pNA), (B) caspase-6 activity (Ac-VEID-pNA), (C) caspase-2 activity (Ac-VDVAD-pNA), (D) caspase-1, -4 activity (Ac-YVAD-pNA), (E) caspase-1, -4, -5 activity (Ac-WEHD-pNA), and (F) caspase-8 activity (Ac-IETD-pNA).

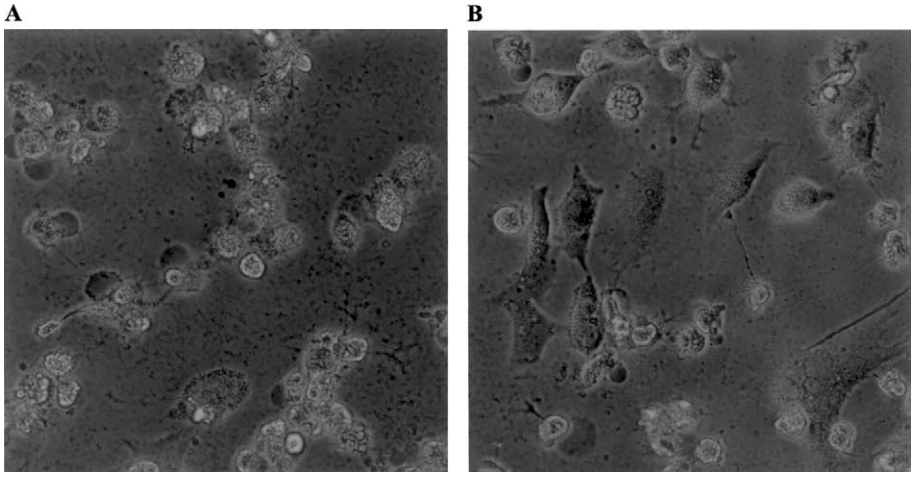

Fig. 5. Inhibition of pierisin-1-induced apoptosis by peptide inhibitor of caspase-3 (Ac-DEVD-CHO). HeLa cells were pre-incubated with 1 mM caspase inhibitor for 1 h. The HeLa cells were then treated with 0.2 ng/ml pierisin-1 and incubated for 24 h. (A) Pierisin-1, (B) pierisin-1+Ac-DEVD-CHO.

caspases such as caspase-3, -6, and -7. Once activated, these effector caspases are directly responsible for the proteolytic cleavage that leads to apoptosis of the cell [7,8]. On the other hand, caspase-2 is considered to be both an initiator and effector caspase [7,8]. We have observed the release of cytochrome *c* from mitochondria into the cytosol and subsequent caspase-9 cleavage in HeLa but not HeLa/bcl-2 cells 8 h after treatment with pierisin-1. Moreover, the caspase substrates Ac-DEVD-pNA, Ac-VEID-pNA, and Ac-VDVAD-pNA, which correspond to caspase-3, -6, and -2, respectively, were efficiently cleaved.

Our findings revealed further caspase-8 to be activated, although only weakly compared with caspase-2, -3, and -6. Caspase-8 is known as an initiator caspase activated by ligation of death receptors, including Fas and TNFR1 through the adaptor molecule FADD that acts in a way distinct from the mitochondrial pathway [7]. However, DNA-damaging agents, such as UV irradiation, or transfected p53, have been reported to activate the Fas receptor system [26–28], indicating the existence of cross talk from upstream of the mitochondrial pathway to the death receptor pathway. Therefore, it is possible that pierisin-1 might also activate caspase-8 as in the case of other agents such as UV irradiation. Taken together, the observations suggest that pierisin-1 induces apoptosis by releasing mitochondrial proteins such as cytochrome *c* into cytosol, followed by initiator and effector caspase activation, with ultimate cleavage of PARP and fragmentation of DNA, similar to the actions of other apoptosis inducers.

Our results suggested that the apoptotic events caused by pierisin-1 occurred mainly via the mitochondrial pathway involving Bcl-2 and caspases. To date, pierisin-1 or pierisin-1-like proteins are only known to exist in *Pieris* butterflies. Does this unique family really induce apoptosis in the cells of *P. rapae* by a mitochondrial pathway involving caspase activation with subsequent DNA fragmentation? Insects have mitochondria and a caspase cascade, and furthermore, cells in tissue undergo apoptosis during metamorphosis. The fact that pierisin-1 accumulates in fifth instar larvae and in the early stage of pupae in *P. rapae* [1,3] might be indicative of a role in metamorphosis. At this stage, however, the precise functions of this protein in *P. rapae* remain unclear and are the subject of ongoing research in our laboratory.

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