Inhibition of Bcl-2-dependent cell survival by a caspase inhibitor: a possible new pathway for Bcl-2 to regulate cell death

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Abstract The REtsAF cell line expresses a temperature-sensitive mutant of the SV40 large tumor antigen. At restrictive temperature (39.5°C), the cells undergo p53-mediated apoptosis, which can be inhibited by Bcl-2. Here, we show that Z-VAD-fmk, a caspase inhibitor, can suppress the Bcl-2-dependent cell survival at 39.5°C. This result suggests that a caspase-like activity can act as an inhibitor of apoptosis in this model, downstream of Bcl-2. Our results also suggest that this activity may be up-regulated by Bcl-2 and may be responsible for cleavage of the tumor suppressor Rb protein.

Key words: Bcl-2; Z-VAD-fmk; Apoptosis; Large T antigen; Rb

1. Introduction

Apoptosis is a process whereby cells activate an intrinsic death program leading to the elimination of unwanted or potentially dangerous cells. The existence of such a program was ascertained through genetic studies in the nematode *Caenorhabditis elegans* that identified genes involved in the cell death program and its control [1]. The *ced-3* and *ced-4* genes are required to the execution of cell death whereas the *ced-9* gene promotes cell survival by inhibiting the death activities of *ced-3* and *ced-4* [2].

CED-3 protein turned out to be a member of a family of cysteine aspartases, known as caspases [3]. These proteases are supposed to form the core of the cell death machinery. All the caspases appear to be synthesized as proenzymes and are proteolytically activated. The upstream 'activating caspases' are able to autoactivate in response to death signals and can activate the downstream 'execution caspases' which would in turn mediate apoptosis by destroying cellular proteins [4]. CED-4 protein interacts directly with CED-3 and promotes its processing and activation [5]. The apoptosis protease-activating factor 1 (Apaf-1) would be a mammalian equivalent of CED-4 [6]. CED-9 protein is homologous to the mammalian Bcl-2 family which contains members that inhibit apoptosis, such as Bcl-2 or Bcl-x_L [7]. It has been shown in both worm and mammalian cells that the anti-apoptotic members of the Bcl-2 family act upstream of the 'execution caspases', somehow preventing their proteolytic processing into active killers [2,8].

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Abbreviations: LT, large T antigen; Tet, tetracycline

In order to better understand the mechanisms that govern cell death versus cell survival, we used a rat embryo fibroblast cell line (REtsAF), conditionally immortalized with a temperature-sensitive mutant (tsA58) of the simian virus 40 large tumor antigen (LT). At permissive temperature (33°C), LT immortalizes these rodent cells via the inhibition of p53 and Rb tumor suppressor proteins [9,10]. At restrictive temperature (39.5°C), inactivation of LT leads to p53 activation and apoptosis of the REtsAF cells [11]. It has previously been shown that apoptosis of REtsAF cells is inhibited by Bcl-2 [12]. In an attempt to better understand the relationships between the anti-apoptotic Bcl-2 and caspases to regulate cell death, we used caspase inhibitors (Z-VAD-fmk, Z-DEVD-fmk and YVAD-cmk) on REtsAF cells conditionally expressing Bcl-2. Here, we show that the Bcl-2-mediated survival of REtsAF cells at 39.5°C involves at least one Z-VAD-fmk-sensitive protease. Furthermore, the accumulation of the truncated Rb protein in *bcl-2*-expressing cells raises the possibility that this protease is responsible for Rb cleavage.

2. Materials and methods

2.1. Cell lines and cell culture

The isolation of the REtsAF cell line and the development of the P1-Bcl-2 cell line have been described previously [12,13]. The cells were propagated at 33°C in Dulbecco's modified Eagle's medium (DMEM) supplemented with penicillin (100 μg/ml), streptomycin (100 U/ml) and 10% fetal calf serum (Gibco). The P1-Bcl-2 cells were routinely maintained in the presence of tetracycline (2 μg/ml) to inhibit the expression of exogenous *bcl-2*. The P1-Bcl-2 cells were propagated for 10 days without tetracycline in the culture medium, in order to allow accumulation of the Bcl-2 protein.

2.2. Caspase inhibitor treatments

The cells were seeded in 60 mm dishes and shifted to 39.5°C, or incubated with staurosporine (0.5 μM) at 33°C, when they reached 50% confluence. Z-VAD-fmk (100 μM), Z-DEVD-fmk (100 μM) and YVAD-cmk (300 μM) were added just before the shift of temperature or staurosporine addition. The percentage of surviving cells was evaluated by determining the proportion of attached cells (estimated by the crystal violet method), expressed as a percentage of the zero time population. Pictures of the cells were taken after 48 h at 39.5°C under a Nikon TMS microscope equipped with a Nikon F601 camera.

2.3. Western blot analysis

The cells were seeded in 100 mm dishes and shifted to 39.5°C when they reached 50% confluence. At different times, cells were rinsed in cold PBS, collected with a scraper and frozen at −80°C. Proteins (80 μg) were separated by SDS-page (in 7.5% acrylamide 0.1% bisacrylamide) and transferred to PVDF membranes (Boehringer Mannheim) according to Towbin et al. [14]. Blots were exposed to the mouse anti-Rb antibody (G3-245, Pharmingen) overnight at 4°C, rinsed in PBS/Tween 20 at 0.5% and exposed for 1 h, at room temperature, to horseradish peroxidase-conjugated anti-mouse immunoglobulin serum (Biosystem). The immunoreactivity was revealed using the Amersham ECL kit.

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3. Results

3.1. Bcl-2-dependent cell survival at 39.5°C is suppressed by Z-VAD-fmk, a caspase inhibitor

We had previously developed the P1-Bcl-2 cell line which is derived from REtsAF cells and expresses the human *bcl-2* gene, under control of tetracycline [12,15]. When tetracycline (Tet) is added to the culture medium, expression of exogenous *bcl-2* is repressed and the cells behave like untransfected REtsAF cells. After tetracycline withdrawal, *bcl-2* is overexpressed and protects the cells from apoptosis at 39.5°C [12]. Bcl-2, like other members of its family, can inhibit apoptosis by preventing activation of caspases (for review see [16]). In order to further examine the relationships between Bcl-2 and caspases, we treated the P1-Bcl-2 cells with caspase inhibitors. We first used Z-VAD-fmk, a broad-spectrum caspase inhibitor which inhibits apoptosis in many models [17,18].

Comparison between P1-Bcl-2 cells with Tet and without Tet at 39.5°C (Fig. 1) confirms that the cells are protected from apoptosis when exogenous *bcl-2* is expressed (−Tet). Unexpectedly, the Z-VAD-fmk treatment of P1-Bcl-2 cells with or without Tet does not protect against apoptosis, but accelerates cell death (Fig. 1). Fig. 1 even shows that addition of Z-VAD-fmk totally abolishes the cell survival brought about by the expression of exogenous *bcl-2*. This result is very surprising because Z-VAD-fmk is an inhibitor of caspases, which are killer proteases. Thus, we tried to determine if Z-VAD-fmk was toxic for the cells.

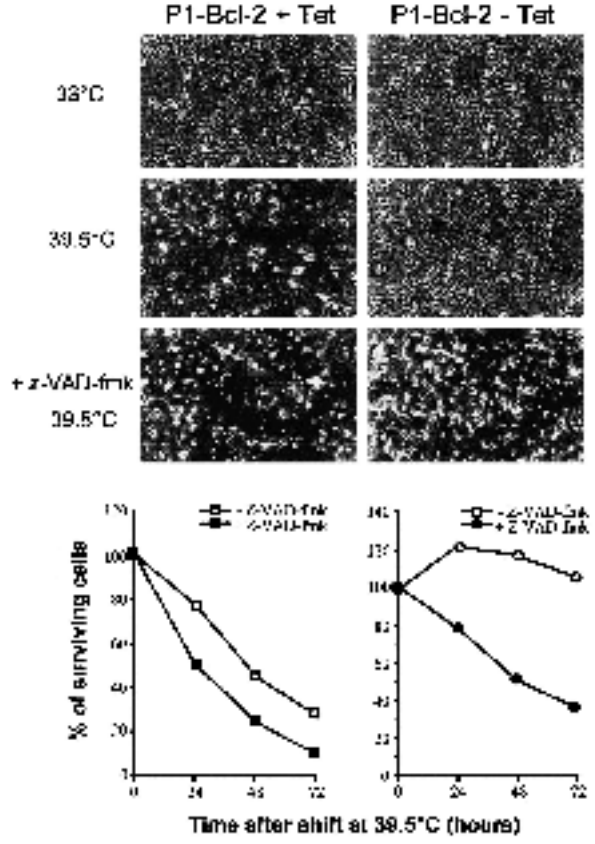

Fig. 1. Bcl-2-dependent survival is suppressed by Z-VAD-fmk, a caspase inhibitor. P1-Bcl-2 cells plus tetracycline and P1-Bcl-2 cells without tetracycline were treated with Z-VAD 100 μM or not treated just before the shift of temperature. Z-VAD-fmk accelerates death of cells with and without Tet, and abolishes the survival effect of *bcl-2* overexpression in cells without Tet. Pictures of the cells were taken at 48 h of treatment.

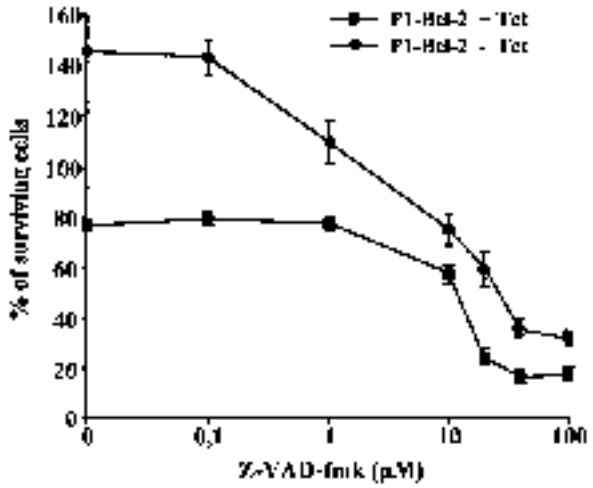

Fig. 2. Influence of Z-VAD-fmk concentration on cell survival at 39.5°C. P1-Bcl-2 cells with or without Tet were incubated for 24 h with different concentrations of Z-VAD-fmk: 0–100 μM. Death of the cells with or without Tet increases in a dose-dependent manner showing that the action of Z-VAD-fmk is specific.

Incubation of P1-Bcl-2 cells with or without Tet with Z-VAD-fmk (100 μM), for 72 h at 33°C, does not change the viability of the cells (data not shown). Moreover, we incubated a cell line obtained from REtsAF, termed Rev-tsAF, with Z-VAD-fmk at 39.5°C. In this cell line, LT lost its temperature sensitivity and the cells were immortalized at 33°C and 39.5°C. We observed that Z-VAD-fmk did not kill the Rev-P1 cells at 39.5°C (data not shown). Taken together, these results show that Z-VAD-fmk is not toxic for the cells. Next, we incubated the P1-Bcl-2 cells with different concentrations of Z-VAD-fmk for 24 h at 39.5°C. Fig. 2 shows that survival of cells with or without Tet decreases in a dose-dependent manner, suggesting that the action of Z-VAD-fmk is specific.

Taken together, these results raise the possibility that a Z-VAD-fmk-sensitive protease activity negatively regulates apoptosis in this system. Since Z-VAD-fmk totally abolishes the effect of *bcl-2* overexpression, we also postulated that this protease activity would occur downstream of Bcl-2 to inhibit apoptosis. The fact that cell death is also accelerated by Z-VAD-fmk when expression of the exogenous *bcl-2* is repressed (+Tet) (Figs. 1 and 2) may be due to the inhibition of the endogenous Bcl-2-dependent cell survival.

3.2. The pro-apoptotic effect of Z-VAD-fmk at 39.5°C is related to its peptide sequence

To further examine the hypothesis of a protease as a mediator of Bcl-2-dependent cell survival, we verified the specificity of Z-VAD-fmk action, compared to other caspase inhibitors. A synthetic caspase inhibitor contains a peptide recognition element, corresponding to those found in endogenous substrates, as well as a chemical function such as aldehyde (CHO) for the reversible inhibitors, or chloromethylketone (cmk) and fluoromethylketone (fmk) for the irreversible inhibitors [19]. To perform this analysis, we used the Z-DEVD-fmk

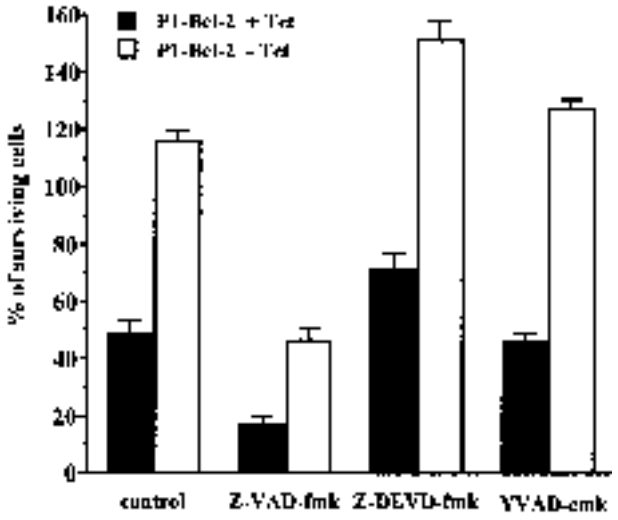

Fig. 3. Effects of different caspase inhibitors on cell survival at 39.5°C. P1-Bcl-2 cells with or without Tet were incubated for 48 h at 39.5°C with the caspase inhibitors Z-VAD-fmk, Z-DEVD-fmk or YVAD-cmk, or were not treated. Z-VAD-fmk increases cell death of cells with and without Tet whereas the Z-DEVD-fmk inhibits cell death. YVAD-cmk has no significant effect on cell survival. The different activities of Z-VAD-fmk and Z-DEVD-fmk demonstrate that the pro-apoptotic effect of Z-VAD-fmk is related to its peptide sequence.

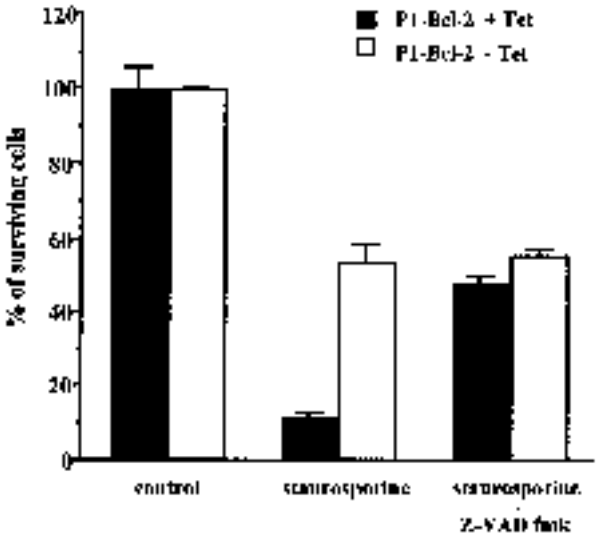

Fig. 4. Influence of Z-VAD-fmk on apoptosis induced by staurosporine. P1-Bcl-2 cells with or without Tet were incubated for 48 h with the caspase inhibitor Z-VAD-fmk, or were not treated, just before the addition of staurosporine (0.5 μM). The Z-VAD-fmk inhibitor increases cell survival of cells with Tet but has no effect on cells without Tet, in contrast to apoptosis at 39.5°C.

and the YVAD-cmk inhibitors, which are both irreversible but present distinct inhibitory spectra. Z-DEVD-fmk preferentially inhibits execution caspases [19], whereas YVAD-cmk inhibits the caspase-1 family, which is involved in interleukin-1β maturation [3].

Fig. 3 indicates that Z-DEVD-fmk and Z-VAD-fmk have opposite effects, showing that the action of Z-VAD-fmk is specific and related to its peptide sequence. This result also suggests that Z-VAD-fmk acts on an anti-apoptotic protease which does not belong to the execution caspase family.

3.3. Action of Z-VAD-fmk is dependent on the cell death inducer

A pro-apoptotic effect for Z-VAD-fmk contradicts data of the literature, in which Z-VAD-fmk inhibits activation of execution caspases and apoptosis. In order to determine the origin of this difference, we induced apoptosis in P1-Bcl-2 cells at 33°C with staurosporine. It has been shown that staurosporine-induced apoptosis is inhibited by Z-VAD-fmk [17].

Fig. 4 shows that the cell death induced by staurosporine in P1-Bcl-2 cells at 33°C is inhibited by both Bcl-2 and Z-VAD-fmk. As expected, Bcl-2 and Z-VAD-fmk apparently have overlapping activities because addition of Z-VAD-fmk on P1-Bcl-2, overexpressing Bcl-2 (−Tet), does not increase the cell survival. These results are in agreement with the roles of Bcl-2 and Z-VAD-fmk reported in the literature and show that the pro-apoptotic action of Z-VAD-fmk is probably related to the death signaling pathway activated by p53 at 39.5°C.

3.4. Evidence for cleavage of a cellular protein up-regulated by Bcl-2 and inhibited by Z-VAD-fmk

The hypothesis of a protease mediating cell survival downstream of Bcl-2 suggests that Bcl-2 could activate this protease and enhance the production of some truncated cellular substrate that favors cell survival. We studied the status of proteins that regulate apoptosis, such as Bax, Bcl-2 or Bcl-x_L, and proteins that regulate cell cycle progression, such as p53, Waf-1 and Rb, in REtsAF and P1-Bcl-2 cells. Among these proteins, we observed that Rb can be a target of such a protease.

A Western blot analysis of the tumor suppressor Rb protein, during kinetics at 39.5°C in P1-Bcl-2 cells with or without Tet, reveals several forms of Rb in cellular extracts (Fig. 5). These forms of Rb are the hypo- (p105)/hyper- (p110) phosphorylated forms, the truncated p100 form described by Chen et al. [20] and the truncated p68 form, identified by An and Dou [21], which is truncated in the N-terminus of Rb.

Unlike p100, the p68 truncated form of Rb is present in P1-Bcl-2 cells (−Tet) overexpressing *bcl-2* but undetectable in P1-Bcl-2 cells with Tet. In order to verify that tetracycline did not suppress p68 production on its own, we added tetracycline to

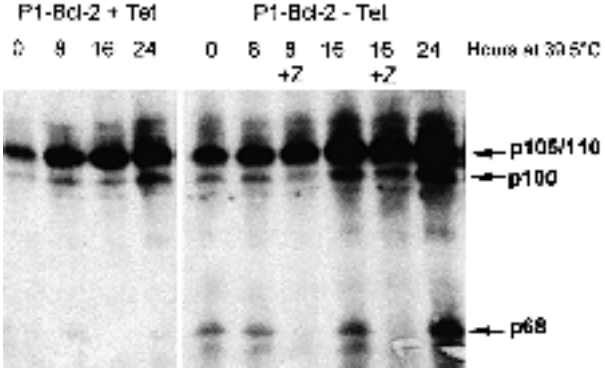

Fig. 5. Bcl-2 activates the cleavage of the Rb protein. P1-Bcl-2 cells with Tet and P1-Bcl-2 cells without Tet were incubated at 39.5°C for different times. Treatment with Z-VAD-fmk was done just before the shift at 39.5°C, where indicated (Z). This Western blot analysis reveals that the production of the truncated p68 form of the Rb protein increases with *bcl-2* expression and is inhibited by Z-VAD-fmk.

another tsA58/LT-immortalized cell line (REtsA15) which survives at 39.5°C and naturally produces p68 at 33°C and 39.5°C. No difference was obtained in p68 production between REtsA15 cells without or with Tet (data not shown). Since p68 production is inhibited by Z-VAD-fmk, these results show that Bcl-2 is actually able to activate, directly or indirectly, a Z-VAD-fmk-sensitive protease which is involved in Rb cleavage and p68 production. The fact that p68 is detected at 33°C, specifically in P1-Bcl-2 cells without Tet, reinforces the idea that Bcl-2 itself regulates the corresponding protease activity, independently of p53 activation at 39.5°C. Furthermore, the accumulation of p68 in *bcl-2*-expressing cells raises the possibility that the Bcl-2-regulated anti-apoptotic protease is responsible for cleavage of Rb.

4. Discussion

The data described above provide evidence of a new pathway for Bcl-2 to inhibit cell death. We have shown that Z-VAD-fmk, a caspase inhibitor, can specifically abolish the survival effect brought about by the overexpression of *bcl-2* in P1-Bcl-2 cells at 39.5°C. Thus, it appears that a caspase-like protein is required downstream of Bcl-2 to mediate its survival effect in this model. Moreover, our results suggest that this protease activity may be up-regulated by Bcl-2 and may be responsible for cleavage of the Rb protein.

The caspase inhibitor Z-VAD-fmk has an anti-apoptotic activity in staurosporine-induced apoptosis and a pro-apoptotic activity in p53-mediated apoptosis at 39.5°C. Thus, we postulate that p53- and staurosporine-induced apoptosis do not involve the same pattern of caspases, and/or the same roles for caspases in cell death. Data in the literature about the relationships between p53-induced apoptosis and the role of caspases are contradictory. Some studies report that p53-dependent apoptosis involves activation of caspases [22] whereas others show that it can occur independently of execution caspases [23]. The fact that a broad-spectrum caspase inhibitor (Z-VAD-fmk) has the ability to kill the P1-Bcl-2 cells at 39.5°C suggests that a death signal, independent of caspases, exists in this system. This signal could be antagonized by Bcl-2 in a pathway requiring the putative protease.

Whether a protease favors cell death or cell survival depends on its targets. In this way, p68 or another substrate truncated by the same protease could mediate the Bcl-2-dependent survival. This hypothesis is consistent with previous reports which show that caspases can act as post-translational modifiers, enhancing some protein activities such as interleukin-1β [24] or the Rb protein [20]. Indeed, the truncated p100 form of Rb has been described as having an enhanced affinity and an increased inhibitory potential for E2F transcription factors [20,25]. Since E2F-1 has the ability to induce apoptosis on its own [26], or in cooperation with p53 [27], E2F-1 may be involved in triggering apoptosis in our model, and p68 may have particular properties to counteract these apoptotic effects.

Another key component of the possible new pathway for Bcl-2 to inhibit apoptosis could be the cell growth and tumor suppressor PML, which is a mediator of multiple apoptotic signals. Indeed, PML death has recently been reported to be accelerated by Z-VAD-fmk [28]. Unfortunately, no connections between PML and Bcl-2 have been established yet. But the information that we have collected concerning the properties and the regulation of the activity of such a protease could facilitate its identification.

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