Possible Involvement of Rapamycin-Sensitive Pathway in Bcl-2 Expression in Human Neuroblastoma SH-SY5Y Cells

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ABSTRACT—In human neuroblastoma SH-SY5Y cells, treatment with immunosuppressants such as FK506, cyclosporin A or rapamycin for 4 days induced the enhancement of the 27-kDa Bcl-2 protein level. Among immunosuppressants, rapamycin has most potency. Treatment with herbimycin A or wortmannin also enhanced Bcl-2 expression, but the BB type of platelet-derived growth factor decreased the level. These results suggest that Bcl-2 expression is probably regulated by the cascade of tyrosine kinase, phosphatidylinositol 3-kinase and rapamycin-sensitive p70 S6-kinase in human neuroblastoma SH-SY5Y cells.

Keywords: Bcl-2, Rapamycin, p70 S6-kinase

Recent exciting findings on apoptosis indicate that both neuronal selection during embryonic development and some forms of neuronal death in adults are regulated by members of the B cell leukemia-2 (Bcl-2) family such as Bcl-2, Bcl-x and Bax (1). Bcl-2 prevents apoptotic death in neurons and several neuron-like cell lines (1). In contrast, Bax promotes apoptosis, probably due to an ability to form heterodimers with Bcl-2 or Bcl-x (2).

The human neuroblastoma cell line SH-SY5Y was subcloned from the SK-N-SH cell line (3). SH-SY5Y cells are often used as a model of human neuronal cells and has been extensively used in the study of neuronal function (4). Recently, it is reported that high levels of Bcl-2 are expressed in SH-SY5Y cells (5). However, the details of the regulatory mechanisms of Bcl-2 protein expression in human neuronal cells are yet unknown. Recently, we determined that Bcl-2 expression was regulated positively by protein kinase C and negatively by protein kinase A (6). In the present study, we further examined the effects of inhibitors for protein phosphatases, tyrosine kinases and phosphatidylinositol (PI) 3-kinase on the expression of Bcl-2 or Bcl-x in human neuroblastoma SH-SY5Y cells.

Mouse monoclonal anti-human Bcl-2 antibody (M887) from Dako (Copenhagen, Denmark); rabbit anti-human Bcl-x antibody (B22630) from Transduction (Lexington, KY, USA); rabbit anti-human Bax antibody (N-20) from Santa Cruz Biotechnology (Santa Cruz, CA, USA); enhanced chemiluminescent detection (ECL) kit from Amersham (Buckinghamshire, England); rapamycin from Biomol (Plymonth Meeting, PA, USA); BB type of platelet-derived growth factor (PDGF-BB) from Oncogene Science (Uniondale, NY, USA); okadaic acid, wortmannin and herbimycin A from Wako (Osaka); phorbol 12-myristate 13-acetate (PMA) from Sigma (St. Louis, MO, USA) were used. FK506 and cyclosporin A were kindly donated by Fujisawa Pharmaceuticals (Osaka) and Sandoz (Basle, Switzerland), respectively.

Human neuroblastoma SH-SY5Y cells were cultured in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% (v/v) fetal calf serum and 50 µg/ml of penicillin and 100 µg/ml of streptomycin and were kept at 37°C in humidified 5% CO₂ / 95% air. The SH-SY5Y cells were treated for 4 days with okadaic acid (at 10 pM - 3 nM), rapamycin (at 30 nM - 3 pM), cyclosporin A (at 0.3 µM - 10 µM), FK506 (at 0.3 µM - 3 µM), wortmannin (at 1 nM - 10 µM), herbimycin A (at 1.74 µM), PDGF-BB (at 3.3 nM), or PMA (at 10 nM). After treatment, cells were rinsed with phosphate-buffered saline and then were scraped and lysed. Subsequently, cell lysates were subjected to immunoblot analysis.

The cell lysate sample (10 µg protein) was dissolved in Laemmli’s sample buffer and subjected to SDS-PAGE (14% polyacrylamide gels) (7). Western blotting was performed by transferring proteins from a slab gel to a...
sheet of polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Richmond, CA, USA) by electroelution at a constant voltage of 50 V for 2 hr at 4°C. After the PVDF membrane was incubated with Tris-buffered saline (pH 8.0) containing 0.05% Tween-20 (TBS-T) and 5% dehydrated skim milk (Difco, Detroit, MI, USA) to block nonspecific protein binding, the membrane was incubated with primary antibodies such as mouse anti-Bcl-2 antibody (diluted 1 : 300) or rabbit antibodies against Bcl-x (1 : 125) and Bax (diluted 1 : 300), and secondary antibody (horseradish peroxidase-linked antibodies against mouse immunoglobulin, diluted 1 : 1500). Subsequently, membrane-bound horseradish peroxidase-labeled antibodies were detected by the ECL system (ECL kit). The protein bands that cross-reacted with antibodies could be detected on X-ray films (X-Omat JB-1; Kodak, Rochester, NY, USA) 5–30 sec after the exposure. The bands of Bcl-2 and Bcl-x on X-ray films were scanned and densitometrically analyzed by a dual-wavelength flying-spot scanner (CS-9000; Shimadzu, Kyoto). Statistical differences were analyzed by Student's t-test. Prestained SDS-PAGE standards (Bio-Rad) were used as molecular weight markers. Apparent molecular weights of phosphorylase B, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor and lysozyme were 105, 82, 49, 33.3, 28.6 and 19.4 kDa, respectively, according to the manufacturer’s instructions.

In SH-SY5Y cells, anti-Bcl-2 antibody immunoreacted mainly with 27-kDa Bcl-2α, and this protein level was increased by treatment of rapamycin or PMA (Fig. 1A). In addition, these enhancements were necessary for treatments of over 2 days (data not shown). Although the 55-kDa protein also immunoreacted with anti-Bcl-2 antibody, this protein level was not changed by any reagents used in this study (Fig. 1A). Therefore, we examined changes in the protein level of 27-kDa Bcl-2α by treatment with several reagents for 4 days. Table 1 shows effects of reagents used in the present study. Although cell toxicity occurred with over 10 nM of okadaic acid, treatments with other reagents at the concentrations used in this study did not cause morphological changes (data not shown).

Okadaic acid is the most potent among reagents used in the present study. Briefly, treatment with okadaic acid for 4 days enhanced the Bcl-2 level in a concentration-dependent manner (EC50 value, 1.8 nM; maximal activity, 422%). Among the other reagents, rapamycin has more potency to enhance the Bcl-2 level (Fig. 2A). Although cyclosporin A and FK506 tended to enhance the Bcl-2 level (Fig. 2A). Although cyclosporin A and FK506 tended to enhance the Bcl-2 level, these enhancements were not significant. EC50

![Fig. 1. Representative immunoblot results using antibodies against Bcl-2 and Bcl-x. SH-SY5Y cells were treated for 4 days with vehicle (lane 1), 10 nM PMA (lane 2) and 3 nM rapamycin (lane 3). After treatments, cells were scraped and lysed. Subsequently, each sample (10 µg protein/lane) was subjected to immunoblotting of antibodies against Bcl-2 (A) and Bcl-x (B). Arrows indicate 27 kDa Bcl-2α in panel A and 29/30 kDa Bcl-xL in panel B.](image-url)
values of rapamycin and wortmannin were 0.11 pM and 1.7 μM, respectively (Fig. 2A). Herbimycin A at 1 μg/ml (1.74 μM) also enhanced it significantly (Table 1). On the other hand, PDGF-BB at 100 ng/ml (3.3 nM) inhibited Bcl-2 level significantly (Table 1).

We further examined effects of PMA and rapamycin on Bcl-x expression. Anti-Bcl-x antibody specifically immunoreacted with a doublet of 29/30-kDa Bcl-xL in SH-SYSY cells (Fig. 1B). Treatment with PMA at 10 nM for 4 days significantly enhanced Bcl-xL level, although treatment with rapamycin at 3 μM did not (Fig. 2B). On the other hand, treatment for 4 days with PMA at 10 nM or rapamycin at 3 μM did not significantly change the protein level of 21-kDa Bax (data not shown).

Previously, we determined that Bcl-2 expression was regulated positively by protein kinase C and negatively by protein kinase A (6). In the present study, since treatment with okadaic acid, a potent inhibitor for protein phosphatases 1 and 2A (8), induced most enhancement of Bcl-2 level, the dephosphorylation by protein phosphatase 1 or 2A also plays key role in the maintenance of Bcl-2 protein level in SH-SYSY cells.

Protein phosphatase 2B (calcineurin) is known as another protein phosphatase and is inhibited by several immunosuppressants such as FK506 and cyclosporin A (9). In brief, FK506 and cyclosporin A bind to FK506-binding proteins (FKBP) or cyclophilins and then inhibit the phosphatase activity of calcineurin (9). Rapamycin also can bind to FKBP, but does not inhibit calcineurin. Recently, several papers described that FK506 and cyclosporin A induced enhancement of Bcl-2 expression but rapamycin and okadaic acid did not in human Burkitt’s lymphoma (BL) cell line (10) and that interleukin-2-induced Bcl-2 expression was inhibited by rapamycin but was not by FK506 in murine bone marrow-derived pro-B (F7) cell line (11). In the present study, however, rapamycin has a greater potency for enhancing the Bcl-2 level than FK506 and cyclosporin A in human neuroblastoma SH-SYSY cells. Thus, regulation of Bcl-2 expression seems to be different between humans and mice in immune-derived cells and between immune cells and neuronal cells in human-derived cells. In SH-SYSY cells, regulation of Bcl-2 expression by calcineurin might be less than that by protein phosphatase 1 or 2A. However, a high concentration of cyclosporin A enhanced Bcl-2 to

Table 1. Effects of treatments with several reagents on Bcl-2α protein levels in SH-SYSY cells

<table>
<thead>
<tr>
<th>Reagent</th>
<th>n</th>
<th>Concentration</th>
<th>% of vehicle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Okadaic acid</td>
<td>4</td>
<td>3 nM</td>
<td>422±98*</td>
</tr>
<tr>
<td>Rapamycin</td>
<td>4</td>
<td>3 μM</td>
<td>189±23**</td>
</tr>
<tr>
<td>Cyclosporin A</td>
<td>4</td>
<td>10 μM</td>
<td>185±40</td>
</tr>
<tr>
<td>Wortmannin</td>
<td>3</td>
<td>10 μM</td>
<td>168±4*</td>
</tr>
<tr>
<td>Herbimycin A</td>
<td>4</td>
<td>1 μg/ml</td>
<td>160±21*</td>
</tr>
<tr>
<td>FK506</td>
<td>4</td>
<td>3 μM</td>
<td>138±19</td>
</tr>
<tr>
<td>PDGF-BB</td>
<td>3</td>
<td>100 ng/ml</td>
<td>90±2*</td>
</tr>
</tbody>
</table>

The Bcl-2α protein level (assessed from densitometry) in the vehicle-treatment was taken as 100%. Each value is the mean±S.E.M. of three determinations. Significances: *P<0.05, **P<0.01 vs the vehicle-treatment (100% activity).

Fig. 2. Concentration-dependency curves of Bcl-2α expression and effects of PMA and rapamycin on Bcl-x expression in SH-SYSY cells. A: Concentration-dependency curves of the effects of rapamycin (○), cyclosporin A (△), FK506 (□) and wortmannin (●) on Bcl-2α expression. Each point is the mean of three determinations that varied by less than 10%. B: Effects of treatment for 4 days with 10 nM PMA and 3 μM rapamycin on expressions of Bcl-2α and Bcl-xL. Open column, PMA-treatment. Hatched column, rapamycin-treatment. The protein level (assessed by densitometry) in the vehicle-treated cells was taken as 100%. Each value is the mean±S.E.M. of three determinations. Significances: *P<0.05, **P<0.01 vs the level in the vehicle-treatment (100% activity).
a level similar to that induced by rapamycin. This detailed mechanism was unclear, but there is a possibility that cyclophilins may exist at higher concentration than FKBP in SH-SY5Y cells.

More recent papers suggest that the complex of FKBP and rapamycin further binds to FKBP-rapamycin-associated protein (FRAP) (12) and then inhibits p70 S6-kinase (13). In addition, PDGF-BB-dependent p70 S6-kinase activation is mediated by activation of PI 3-kinase (14) and protein kinase B (c-Akt) (15). In the present study, both the tyrosine kinase inhibitor herbimycin A and the PI 3-kinase inhibitor wortmannin also enhanced the Bcl-2 level, but PDGF-BB decreased it. From these observations, we considered that the activation cascade of tyrosine kinase, PI 3-kinase and p70 S6-kinase further regulates Bcl-2 expression in SH-SY5Y cells. On the other hand, PMA-treatment induced enhancement of both Bcl-2α and Bcl-xL levels. However, rapamycin-treatment did not change the Bcl-xL level. Thus, expression mechanisms of Bcl-2 and Bcl-x seem to be different in SH-SY5Y cells.

In conclusion, the present study suggests that maintenance of Bcl-2 protein level is regulated by protein phosphatase 1 or 2A. In addition, the activation cascade of p70 S6-kinase is also possibly involved in negative regulation of Bcl-2 expression in human neuroblastoma cells. On the other hand, Bcl-x expression was differently regulated than Bcl-2 expression.

Acknowledgments

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REFERENCES

4 Wojcikiewicz RJH, Tobin AB and Nahorski SR: Muscarinic receptor-mediated inositol 1,4,5-trisphosphate formation in SH-SY5Y neuroblastoma cells is required acutely by cytosolic Ca2+ and by rapid desensitization. J Neurochem 63, 177–185 (1994)