EXPRESSION OF *BCL-2* AND *BCL-X* GENES IN LYMPHOCYTES AND TUMOR CELL LINES DERIVED FROM MDV-INFECTED CHICKENS

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Summary. – To characterize the molecular events involved in both apoptosis and transformation process induced by Marek's disease virus (MDV), the expressions of the *bcl-2* and *bcl-x* genes, ones of the dominant apoptosis-regulating genes, in Marek's disease (MD) tumor cell lines and cells prepared from MDV-infected chickens were analyzed. The expression of *bcl-2* was down-regulated in both $CD4^+$ and $CD8^+$ T cells prepared from MDV-infected chickens at 3 weeks p.i. No *bcl-2* transcript was detected in MD tumor-derived MSB1 and MTB1 cell lines, which had been established from primary MD tumors. On the other hand, the *bcl-x*L transcript whose product can also inhibit apoptosis was expressed in cell lines derived from MD. By the treatment with phorbol 12-myristate 13-acetate (PMA) and ionomycin, normal $CD4^+$ T cells were induced to express *bcl-x*S which can promote apoptosis, while *bcl-x*L was constitutively expressed in MD cell lines. Our results suggest that *bcl-x*L rather than *bcl-2* might play an important role in the transformation process by MDV.

Key words: apoptosis; bcl-2; bcl-x; MDV

Introduction

MDV is a pathogenic agent of MD, which is characterized by malignant T cell lymphoma and nerve demyelination. The molecular mechanism(s) of transformation of T cells by MDV has not been completely understood. We have previously reported that, in chickens infected with MDV, $CD4^+$ T cells undergo apoptosis at 2–3 weeks p.i. (Morimura *et al.*, 1995). However, it is also generally accepted that $CD4^+$ T cells are the main target for transformation by MDV (Schat *et al.*, 1991). These observations suggest that the regulatory mechanism of apoptosis could also be involved in the subsequent transformation process by MDV.

Many viruses, including chicken anemia virus, influenza virus and human immuno-deficiency virus-1, have been known to induce apoptosis in infected cells (Hinshaw *et al.*, 1994, Jeurissen *et al.*, 1992), or uninfected cells (Boudet *et al.*, 1996, Li *et al.*, 1995). It has been also reported that several viruses down-regulate *bcl-2* expression in the apoptotic target cells *ex vivo* as well as *in vitro* culture, and this down-regulation may determine the susceptibility to apoptosis (Boudet *et al.*, 1996). The proto-oncogene *bcl-2* was discovered by virtue of its translocation into the immunoglobulin heavy chain locus in human follicular B cell lymphoma (Tsujimoto *et al.*, 1984). The function of the gene product (Bcl-2) is known to inhibit or delay apoptotic cell death (Allsopp *et al.*, 1993). In fact, apoptosis observed in cell lines induced by several viral infection was blocked by Bcl-2 expression (Hinshaw *et al.*, 1994). These observations indicate that Bcl-2 is one of the key involved in the regulation of apoptosis induced by viral infections.

Escape from apoptosis is frequently an essential component of transformation and tumor development induced by some tumor viruses. This escape could provide opportunities for other oncogenes in infected cells to be activated during the survival. For example, LMP1 of Epstein-Barr virus mediates protection from apoptosis by up-regulating expression of *bcl-2* (Henderson *et al.*, 1991). Meq protein of MDV induces transcription of *bcl-2* in Rat-2 cells (Liu *et al.*, 1998). Moreover, herpesvirus saimiri and other lymphotropic herpesviruses code for proteins that are homologous to Bcl-2 (Nava *et al.*, 1997). Thus, the interplay be-

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tween these virus infection and expression of *bcl-2* has important implications for virus persistence and for the pathogenesis of virus-associated malignant disease. Many *bcl-2*-like genes including *bcl-x* and *Bax* have been identified (Kroemer, 1997); they form heterodimer with one another to play a central role in the regulation of apoptosis (Oltvai *et al.*, 1993). Here, to study the role of *bcl-2* gene family in the transformation process by MDV, expression of *bcl-2* and *bcl-x* genes was analyzed in T cells from MDV-infected chickens and in MD tumor cell lines.

Materials and Methods

Experimental chickens, virus and cell lines. Fertile eggs of Shaver 288 White Leghorn chickens were purchased from Hokuren Co. Ltd (Sapporo, Japan). These eggs were hatched, and birds were raised in our laboratory.

A highly virulent strain of MDV, Md5, used for this experiment was propagated in chick kidney cells, and virus titers were determined by plaque assay. Chickens were challenged intramuscularly with 10,000 PFU of strain Md5 at 5 days after hatching.

Chicken cell lines established from chicken lymphomas used in this study were MD cell lines MDCC-MSB1 (Akiyama and Kato, 1974), -MTB1 (Ikuta *et al.*, 1985), -HP1, -HP2 (Powell *et al.*, 1974) and -RP1 (Nazerian *et al.*, 1976), avian leukosis cell lines LSCC-CU10 (Calnek *et al.*, 1978), an reticuloendotheliosis cell line RECC-Ku7 (Sasaki *et al.*, 1981).

Monoclonal antibodies (MAbs) and isolation of CD4$^+$ or CD8$^+$ T cells. The following MAbs were used to isolate T cell subsets: CT4, which can recognize CD4 molecule (Chan *et al.*, 1988) and 11-39, which can recognize CD8 molecule (Luhtala *et al*, 1993). TCR1, which can recognize gdTCR (Chen *et al.*, 1988) was purchased from Southern Biotechnology Associates Inc. (Birmingham, AB).

For isolation of T cell subsets, the spleens were obtained from chickens at 3–5 weeks after MDV-infection. T cell-enriched populations were obtained by filtration of spleen cell suspensions through nylon wool. Nylon wool-passed cells (5 x 10^7) were incubated with 2 ml of the culture supernatant of either CT4 or 11-39 containing 10 μg of TCR1 on ice for 30 mins. After washing with culture medium (RPMI 1640 containing 10% heat-inactivated FCS), cells were incubated with 2 ml of rabbit anti-mouse Ig (Zymed, San Francisco, CA), diluted 1:500 with culture medium, on ice for 30 mins. After washing with culture medium, cells were incubated with 1.5 ml of low toxic rabbit complement (Cedarane, Canada) diluted with culture medium (1:10) at 37°C for 45 mins. Viable cells were recovered by Ficoll-Conray gradient centrifugation. Flow cytometric analysis revealed that the purity of each T cell subset separated by this procedure ranged from 92 to 98%.

Treatment of fractionated T cells and cell lines with PMA and ionomycin. Purified splenic CD4$^+$ or CD8$^+$ T cells were resuspended (1.5 x 10^6/ml) in RPMI 1640 supplemented with 10% heat-inactivated FCS and with or without 10 ng/ml PMA and 150 ng/ml ionomycin. These cells were incubated at 40°C for 48 hrs in a humidified atmosphere containing 5% CO_2 before used for extraction of total cellular RNA. To induce apoptosis in tumor cell lines, they were treated with 10 mg/ml mitomycin C (Kyowahakko, Tokyo, Japan) for 24 hrs.

Northern blot analysis. The *bcl-2* cDNA was amplified from a cDNA library of the chicken spleen by polymerase chain reaction (PCR) using a set of primers, A1 (5'-TCCCCTCGGAAACCATG GCT-3') and A2 (5'-TGAAGTCAC CCAGTTTATCG-3'), designed from the reported sequence (Eguchi *et al.*, 1992). The PCR product was cloned into pGEM-T vector (Promega, Madison WI). The recombinant vector was designated pGEM-*bcl-2*.

Expression of *bcl-2* was analyzed by Northern blot hybridization using a ^{32}P-labeled *bcl-2* cDNA (a *Nco*I fragment of pGEM-*bcl-2*) as a probe. Total cellular RNA was extracted from CD4$^+$ or CD8$^+$T cells with Trizol (Gibco BRL). Samples (5 μg) were electrophoresed and blotted to the Nylon membrane Hybond-N$^+$ (Amersham). The blot was hybridized with ^{32}P-labeled probes, and washed once with 2 x SSC plus 0.1% SDS for 15 mins at room temperature, washed twice with 0.1 x SSC plus 0.1% SDS for 30 mins at 65°C. The blot was then subjected to autoradiography.

Reverse transcription-polymerase chain reaction (RT-PCR). For the detection of the *bcl-x* transcripts, RT-PCR was performed using a set of primers, X1 (5'-TGTGAAAATGTCCAGCA GTA-3') and X2 (5'-ACACAACCATGGGGTC-ACTT-3') designed from the reported nucleotide sequence (Boise *et al.*, 1993). Using these primers, two forms of the *bcl-x* transcripts, the long (*bcl-x*L) and short ones (*bcl-x*S) could be distinguished from each other based on the size of amplified cDNAs.

RT-PCR was done by the method of Takagi *et al.* (1998). Extracted total cellular RNA (2–5 μg) was treated with DNase I. The RT step was performed using 20 U of reverse transcriptase (RAV-2, Takara, Kyoto, Japan) and 200 pmoles of oligo(dT)$_{15}$ at 42°C for 60 mins. Then, newly synthesized first strand cDNA (30 μl) was mixed with 70 μl of distilled water. The PCR step was performed with 10 ml of diluted cDNA under the following conditions: one cycle of 4 mins at 94°C, 35 cycles of 1 min at 94°C, 1.5 min at 55°C, and 1.5 min at 72°C, and final extension cycle of 10 mins at72°C. The PCR products were separated in 2% agarose gel and visualized by staining with ethidium bromide.

Results and Discussion

Down-regulation of bcl-2 transcription in T lymphocyte subsets

In chickens infected with MDV, the number of CD4$^+$ T cells transiently increased at 2 weeks p.i., and then decreased rapidly due to apoptosis (Morimura *et al.*, 1995). However, considerable number of CD4$^+$ T cells escaped from the apoptosis and remained in the periphery, suggesting that these cells were resistant to apoptosis and became targets for transformation by MDV. Thus, transcription of the *bcl-2* gene was analyzed in these remaining CD4$^+$ and CD8$^+$ T cells from MDV-infected chickens (Fig. 1). The *bcl-2* gene product has been identified as one of the factors which can res-

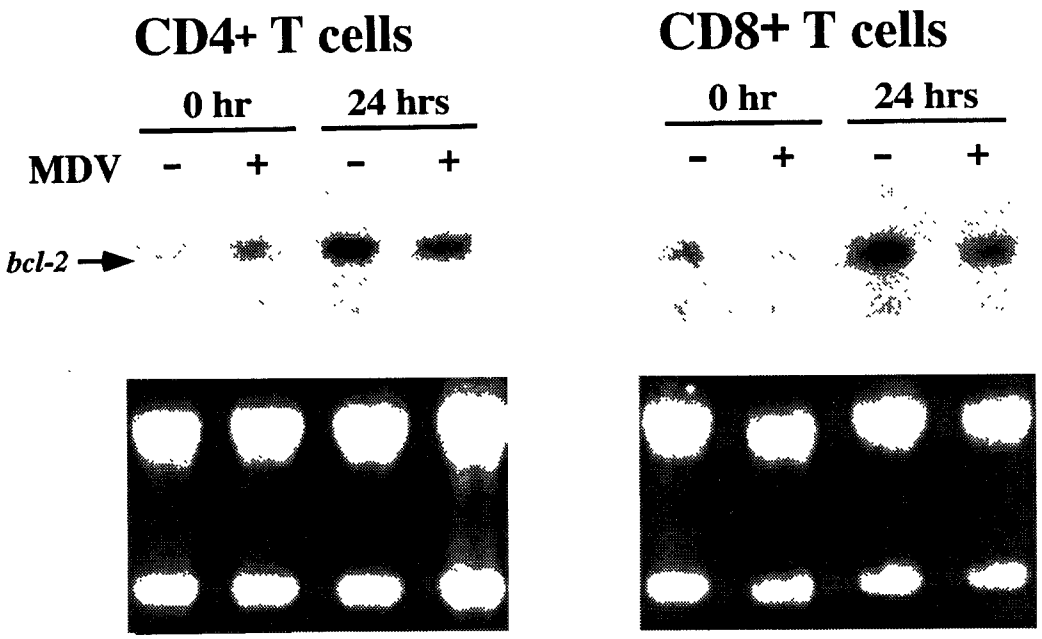

Fig. 1

Northern blot analysis of *bcl-2* gene expression in T cell subsets prepared from MDV-infected chickens

Total cellular RNAs were prepared from $CD4^+$ and $CD8^+$ T cells cultured for 0 and 24 hrs with PMA and inomycin from normal (–) and MDV-infected chickens (+), and hybridized with ^{32}P-labeled *bcl-2*-specific probe (upper panel). Ribosomal RNA on the agarose gel was stained with ethidium bromide to ensure equal amount of loadings (lower panel). Arrow indicates the position of *bcl-2*-specific transcript.

cue cells from apoptosis by several inducers (Allsopp *et al.*, 1993; Garcia *et al.*, 1992; Vaux *et al.*, 1988). The *bcl-2* gene expression slightly decreased in $CD4^+$ T cells from infected chickens. In addition, this gene transcription was drastically suppressed in $CD8^+$ T cells from infected chickens compared to that of age-matched normal chickens. These results suggest that *in vitro* apoptosis in T cell subsets from MDV-infected chickens may be regulated at least partially by the down-regulation of *bcl-2* expression. Up-regulation of the *bcl-2* transcript in T cells was observed *in vitro* immediately after mitogenic stimulation (Boise *et al.*, 1993). However, treatment with PMA and ionomycin failed to up-regulate *bcl-2* expression in T cells from MDV-infected cells. Thus, it was shown that T cells escaping from apoptosis in MDV-infected chickens are also susceptible to apoptosis upon some stimuli.

Expression of the bcl-2 and bcl-x genes in tumor cell lines

Expression of the *bcl-2* gene was down-regulated even in $CD4^+$ T cells which escaped from apoptosis. In the next experiment, we analyzed the expression of *bcl-2* in tumor cell lines derived from MD to determine if the up-regulation of the *bcl-2* is involved in the transformation process induced by MDV. Liu *et al.* (1998) reported that overexpression of Meq protein leads to transformation of Rat-2 cells, and that these proteins protect the transformed cells from apoptosis probably due to transcriptional induction of *bcl-2*. However, we found that the expression of *bcl-2* was under the detectable level in MSB1 (Fig. 2) and MTB1 cell lines (data not shown), which had been established from primary MD tumors. In contrast, *bcl-2* expression was detected in normal $CD4^+$ T cells, and it was up-regulated when these cells were treated with PMA and ionomycin (Fig. 1). These results suggest that up-regulation of *bcl-2* expression may not be prerequisite for subsequent transformation by MDV. It should be noted that a small amount of *bcl-2* RNA was detected in other MD cell lines examined, RP1 (Fig. 2), HP1 and HP2 (data not shown). Since these cell lines had been derived from chickens transplanted with tumor cells and are themselves transplantable, *bcl-2* could be important for progression from low- to high-grade malignant lymphoma (McDonnell *et al.*, 1991).

bcl-x is a member of the *bcl-2* gene family, and can function as a *bcl-2*-independent regulator of apoptosis (Boise *et al.*, 1993). Alternative splicing results in two different *bcl-x* mRNAs, the long (*bcl-x*L) and short forms (*bcl-x*S). *bcl-x*L, similarly to *bcl-2*, inhibits apoptosis whereas *bcl-x*S inhibits the ability of Bcl-2 to enhance the survival of cells, thus

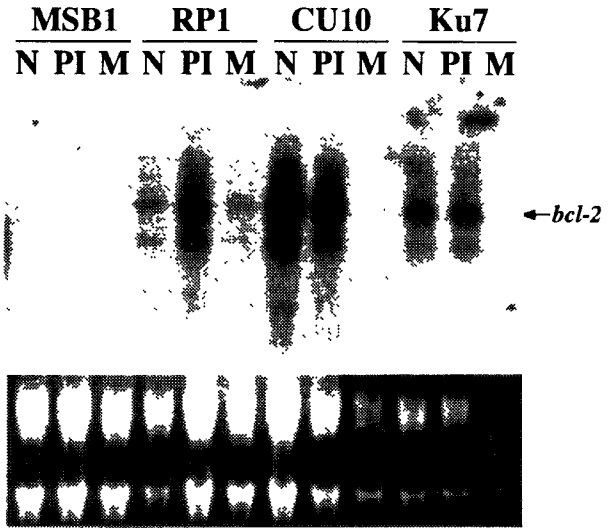

Fig. 2
Northern blot analysis of *bcl-2* gene expression in MD tumor cell lines
Total cellular RNAs were prepared from tumor cell lines either non-treated (N), treated with PMA and inomycin (PI), or with mitomycin C (M), and hybridized with ^{32}P-labeled *bcl-2*-specific probe (upper panel) Ribosomal RNA on the agarose gel was stained with ethidium bromide to ensure equal amount of loadings (lower panel). Arrow indicates the position of *bcl-2* -specific transcript.

promoting apoptosis. In order to analyze the expression of *bcl-x* in MD cell lines, RT-PCR was performed (Fig. 3). In MSB1 and RP1 cell lines, *bcl-x*L was constitutively expressed while *bcl-x*S was induced when control CD4$^+$ T cells were treated with PMA and ionomycin. No *bcl-x*-specific cDNA was amplified from RNA prepared from CU10 or Ku7 cell lines, which had been derived from avian leukosis and reticuloendotheliosis respectively, under the conditions used in this experiment. Thus, the induction of *bcl-x*L expression could contribute to the transformation process by MDV. In T cells infected with MDV, *bcl-x*L may compensate the roles of *bcl-2*, and thus preventing cells from apoptosis so that this prevention can provide many chances for cells to accumulate qualitative and quantitative changes in other oncogenes.

In conclusion, the results obtained here suggest that expression of *bcl-2* might be dispensable for transformation process induced by MDV, and that *bcl-x*L could functionally substitute for *bcl-2* and contribute to this process at least partially. However, apoptosis could also be controlled by other potential factors (*i.e.*, other members of the Bcl-2 family, such as Bad or Bax, Fas-FasL interaction, or several viral proteins). Investigation of the role of these molecules in *ex vivo* and *in vitro* apoptosis of T lymphocytes could be important for understanding the pathogenesis of MD.

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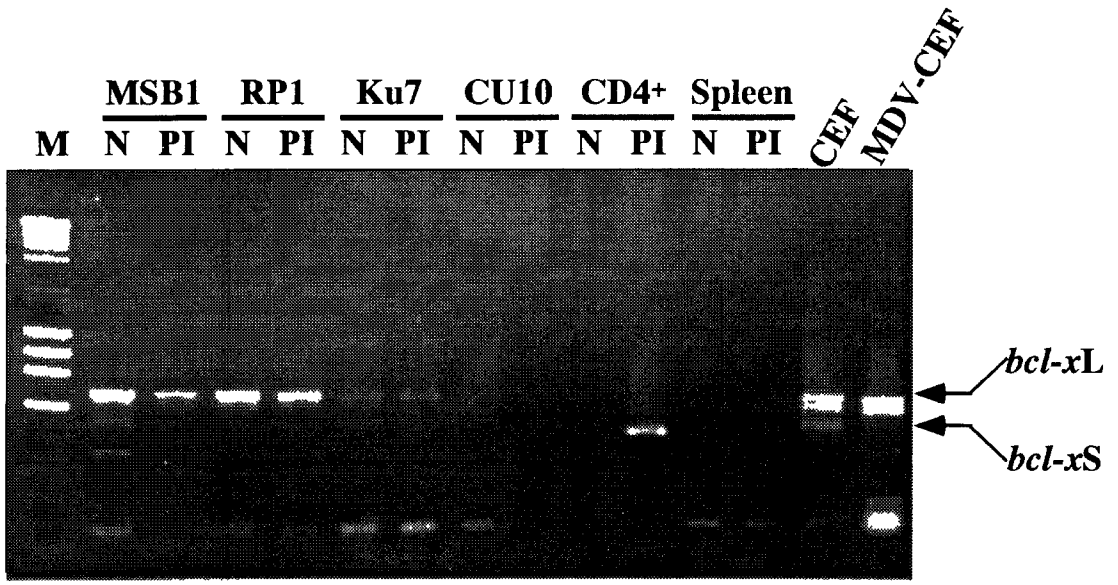

Fig. 3
RT-PCR analysis of *bcl-x* gene expression in MD tumor cell lines
Total cellular RNAs prepared from tumor cell lines were used for PCR analysis. Arrows indicate the positions of the long (*bcl-x*L) and short forms (*bcl-x*S) of *bcl-x* transcripts. RNAs were also extracted from CEF infected with MDV.

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