INHIBITION OF APOPTOSIS BY MAREK'S DISEASE VIRUSES

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Summary. – Strains of Marek's disease virus (MDV), a herpesvirus, have been shown to augment the development of lymphoid leukosis induced by retroviruses, the avian leukosis virus (ALV) or the reticuloendotheliosis virus. In this study we explored the possibility that the ability to augment lymphoid leukosis may be correlated with the ability of different strains of MDV to block apoptosis. Subclones of the ALV-transformed B cell line, DT40, which was free of MDV DNA were infected with either R2/23 strain of MDV-1, SB1 strain of MDV-2, or turkey herpes virus (HVT), a MDV-3. We found that most of the normal DT40 cells and DT40 cells infected with the R2/23 became apoptotic when cultured in serum-reduced medium. By contrast, the frequency of apoptotic cells was greatly reduced in the DT40-SB1 and DT40-HVT subclones. These findings suggest that because the SB-1 strain persists in the ALV-infected cells, it may augment lymphoid leukosis by inhibiting apoptosis and providing a survival advantage to the B cells which have a deregulated *myc* proto-oncogene.

Key words: ALV; Marek's disease; MDV; leukosis; apoptosis

Introduction

Some strains of MDV augment the development of lymphoid leukosis (LL), a B-cell neoplasia induced by infection with the ALV (Bacon *et al.*, 1989.). Co-infection of chicks with non-pathogenic strains of MDV-2 experimentally infected with ALV is associated with an increase in the number of LL tumors per chicken, a higher frequency of metastatic tumors, and an increase in the overall incidence of LL compared to non-MDV-infected chickens. This augmentation of LL was observed in ALV-infected chickens that were co-infected with MDV-2 as late as 6 weeks of age (Fadly and Witter, 1993). MDV-2 has also been shown to augment reticuloendotheliosis virus-induced B-cell lymphomas (Alay, 1996). The mechanism of MDV enhancement of LL is not known.

To understand the mechanism of augmentation it is important to understand the natural history of LL. The development of LL in chickens is marked by well-defined stages (Purchase, 1988; Ewert and DeBoer, 1988) starting with ALV infection of immature B lymphocytes in the bursa of Fabricius, the target cells of transformation. The first evidence of transformation is the appearance of 5–20 enlarged, hyperplastic bursal follicles which are detectable at 4–14 weeks after neonatal ALV infection depending on the line of chickens and strain of ALV. After several weeks, one or two of these hyperplastic follicles progress to become a lymphoma(s); the remaining follicles regress with age (Ewert and DeBoer, unpublished data). In the final stage of the disease (14–25 weeks after infection), cells from the bursal lymphoma(s) migrate to distal organs and the chickens ultimately succumb.

Each transformed follicle and LL tumor contains a clonally expanded population of B cells that have a provirus integrated in the c-*myc* locus (Ewert and DeBoer, 1988; Hayward *et al.*, 1981). The insertion of a strong viral promoter in the c-myc locus results in the deregulation of c-*myc*. Digestion of cellular DNA with restriction enzymes that cut in the provirus and in c-*myc* produced a proviral-*myc* junction fragment for each transformed follicle or LL tumor that varies in size depending on the site of proviral insertion in the c-*myc* locus. The fact that all of the hyperplastic follicles contain a deregulated c-*myc* gene but only a few progress toward malignant tumors suggests a requirement for factors in addition to c-*myc* activation to effect neoplastic transformation.

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We have previously shown that in chickens co-infected with ALV and the SB1 strain of MDV-2, the MDV DNA is present in the bursal and metastatic tumors examined, but not in ALV-infected non-transformed bursal lymphocytes of the same chickens (Fynan, 1992). Based on these findings, we hypothesize that MDV enhancement of ALV-induced B cell lymphomagenesis most likely involves direct influence of MDV on the target cell of ALV transformation at some point during the multi-stage process of lymphoma development.

Preliminary studies indicated that ALV-transformed cell lines which carried the SB1 genome were more resistant to apoptosis induced by serum deprivation than similar lines that were not infected with MDV. Activation of genes, e.g. *bcl-2*, that block apoptosis are associated with tumor progression in neoplasias involving deregulation of the c-*myc* gene (Strasser, 1990). Consequently, we hypothesized that MDV strains may also promote retroviral tumor progression by blocking apoptosis. To test this hypothesis we examined the ability of strains of MDV which differed in their ability to augment LL for blocking apoptosis in ALV-transformed B cell line.

Materials and Methods

Infection of DT40 cells. The ALV-transformed cell line, DT40, which was free of MDV was a gift from Dr. Eric Humphries (Baba *et al.*, 1985). Separate cultures of DT40 cells were infected with three different strains of MDV by co-culturing on monolayers of infected fibroblasts for 24 hrs. Chicken embryo fibroblasts (CEFs) infected with SB1 or HVT MDV were obtained from Intervet Inc. and R2/23-infected CEFs were a gift from Dr. R. Witter. The DT40 cells were aspirated from the fibroblast cultures and subcloned at limiting dilution in 96-well microtiter plates. DNA from 20–30 colonies of DT40 cells was analyzed for MDV DNA by Southern blot analysis. Positive cultures were subcloned and reanalyzed for the respective MDVgenomes.

Apoptosis analysis. DT40 cells were induced to become apoptotic by reducing the serum content of the medium to 0.1%. The apoptotic cells were identified by staining the DNA with acridine orange and examining wet mounts of cells by fluorescence microscopy. The nuclear chromatin gave a green fluorescence which was normally diffuse in the nucleus but was typically highly condensed in the apoptotic cells. Cells were also analyzed by *in situ* end labeling of fragmented DNA with dUTP-FITC using terminal deoxynecleotidyl transferase followed by flow cytometry analysis (TUNEL assay) (Gravrieli, 1992).

Results and Discussion

The objective of this study was to test the hypothesis that some strains of MDV could augment LL development by inhibiting apoptosis. Preliminary studies had indicated that ALV-transformed B cell lines that carried SB1 MDV were more resistant to apoptosis than lines that were free of MDV. But it was not known if other strains of MDV that did not augment LL could also inhibit programmed cell death. Also, we could not be sure that the effect was cell line-specific and independent of MDV. Therefore a single cell line was chosen for this study to eliminate cell line specific variables. The DT40 cell line had been established from an ALV-transformed lymphoma and was free of MDV (Baba *et al.*, 1985). Also DT40 had been characterized as being typical of bursal B cells both with respect to immunoglobulin gene conversion (Buerstedde, 1990) and cell surface antigen phenotype (unpublished data), and therefore would be representative of the transformed bursal cells that are affected by the target of MDV augmentation. Furthermore, the DT40 cells could be induced to undergo apoptosis by reducing serum in the medium, with more than 60% of the cells becoming apoptotic within 24 hrs, as evidenced by condensation of nuclear chromatin and cell shrinking.

Infection of the DT40 cells was accomplished by co-cultivation on MDV-infected fibroblast cultures. Three non-pathogenic stains of MDV were chosen for the analysis. SB1, a widely used vaccine MDV-2 strain which had been shown to augment LL; HVT, a MDV-3 strain which did not augment LL; and R2/23, a MDV-1 strain that did not augment LL (R. Witter, personal communication). The transfer of infection to the DT40 cells was relatively inefficient with about 5% of the cultures becoming infected. However, once infected, the presence of MDV could be confirmed in the DT40 cells during the six months of the study as evidenced by detection of the cell-associated MDV DNA. These DT40-MDV sublines enable direct comparisons of gene expression and of growth characteristics between cells of the same genetic and phenotypic background which differ in the presence of the respective MDV genomes. Although the SB1 and HVT viruses had no noticeable effect on the growth of the DT40 cells, the R2/23 strain increased the cell doubling time by about 30%.

To test the effect of the different stains of MDV on apoptosis the DT40 cells were cultured in medium that was serum-reduced. Two types of assays were used to analyze apoptosis. Acridine orange is used to give a quick microscopic assessment of nuclear chromatin condensation typical of apoptotic cells. The end-labeling assay is slightly more sensitive because it detects DNA fragmentation that precedes the chromatin condensation. As shown in Table 1, at 24 hrs following serum deprivation, between 78 and 90% of the cells were apoptotic. The R2/23 strain had only a slight effect on apoptosis whereas both the SB1 and HVT strains dramatically reduced apoptosis of the DT40 cells. By comparison, the RAV-1-transformed cell line 740 that came from an SB1-infected chicken, was also resistant to apoptosis under the same conditions. No essential difference was observed between independent DT40 subclones of the same virus.

Table 1. Inhibition of apoptosis in DT40 cells by different strains of MDV

Cell line	Percentage of apoptotic cells	
	Acridine orange assay	TUNEL assay
DT40	78%	97%
DT40-R2/23, MDV-1	66%	87%
DT40-SB1, MDV-2	12%	3%
DT40-HVT, MDV-3	18%	23%
740, (SB1$^+$ALV lymphoma)	5%	1%

These data demonstrate that MDV stains differ in their ability to inhibit apoptosis of ALV transformed B cells. However, the correlation with LL augmentation and blocking of apoptosis is not consistent, since HVT does not augment LL but does inhibit apoptosis. The reason why HVT may not influence LL is that it does not persist in the B cells as does the SB1 (unpublished data). Chickens infected *in ovo* with ALV and vaccinated at hatch with HVT and SB1 developed LL tumors which contained SB1 but not HVT. Therefore since HVT did not persist in the B cells the virus could not influence the development of LL. We therefore conclude that strains of MDV that inhibit apoptosis may promote tumor progression provided they persist in the target cell population. In the case of LL the target population is bursal B cells. Since most bursal B cells undergo apoptosis before they leave the environment of the bursa of Fabricius, the ability of bursal cells to survive outside the bursal environment is normally associated with maturation of the B cell to a post bursal B cell. This process of maturation is blocked by activation of the *myc* oncogene in ALV- or REV-transformed cells by maintaining the cells in cycle. Consequently, a secondary event, e.g. proviral activation of cellular genes may be required to cause the pre-neoplastic cells to become independent of the growth factors in the bursa and to metastasize. Strains of MDV that augment LL may provide such survival factors by blocking apoptosis which permit transformed B cells to metastasize from the bursal environment.

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