Expression of Murine Leukemia Virus Envelope Protein Is Sufficient for the Induction of Apoptosis[▽]

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The generation of cytopathic effects by murine leukemia viruses (MLVs) in different cell types correlates with the ability of the virus to induce thymic lymphoma. We showed that the induction of apoptosis in mink epithelial cells by mink cell focus-forming (MCF) MLV infection results in the accumulation of high levels of both unintegrated viral DNA and the envelope precursor glycoprotein (gPr80^{env}). Comparisons of envelope protein expression levels of plasmid clones of the *env* gene of the MCF13 and noncytopathic NZB-9 MLV strains demonstrated that the accumulation of MCF13 gPr80^{env} results in endoplasmic reticulum stress and is sufficient for the induction of apoptosis.

The pathogenicity of some retroviruses correlates with their ability to induce cytopathic effects involving different cell types (1, 2, 8, 16, 22, 24, 25, 27, 29, 30, 34, 35). Diseases induced by cytopathic retroviruses include malignancies, immunodeficiency, and neurodegeneration. An example of a cytopathic murine leukemia virus (MLV) that induces thymic T-cell lymphoma is the mink cell focus-forming (MCF) MLV (5, 20, 32). Upon characterizing the early events that occur during the development of thymic lymphoma induced by inoculation of the MCF13 MLV strain into AKR mice, we detected a significant reduction of thymic lymphocytes via apoptosis (36). To better understand this phenomenon, we established an *in vitro* culture system utilizing CCL64 mink epithelial cells, which similarly undergo apoptosis after infection with MCF13 MLV (18, 35).

It has been demonstrated for several cytopathic retroviruses that there is a strong correlation between virus superinfection and cell killing (4, 12, 13, 16, 17, 23, 33, 35). One of the results of retroviral superinfection is the accumulation of high levels of unintegrated viral DNA in cells, which has been implicated in the induction of cytopathic effects (4, 12, 13, 17, 23, 33, 35). An additional consequence of superinfection by some pathogenic retroviruses, including MCF13 MLV, is the accumulation of high levels of the envelope precursor glycoprotein (gPr80^{env}) in the endoplasmic reticulum (ER), which results in ER stress and apoptosis (7, 14, 15, 19, 21, 30). Previous studies of superinfection related to cell killing involved virus infection of cells, which results in the production of high levels of both unintegrated viral DNA and the envelope protein; thus, no conclusions could be drawn about whether both viral products are essential for cell killing or whether only one of them is sufficient. Because of our previous detection of the accumulation of high levels of gPr80^{env} in cells that are undergoing apoptosis by MCF13 MLV infection (19), we undertook this

study to determine whether cell killing is inducible by the envelope protein alone and whether ER stress is involved.

For exogenous expression of the MCF13 MLV envelope protein, we produced a plasmid clone consisting of the *env* gene of this retrovirus. Because we previously observed that the xenotropic MLV strain NZB-9 did not induce either apoptosis or ER stress in virus-infected cells (18, 19), we also cloned the *env* gene of this virus for comparison. The envelope glycoproteins of MCF13 and NZB-9 MLV were expressed in 1.6×10^6 mink epithelial cells by transient transfection of 25 μ g of plasmid DNA with the use of Lipofectamine 2000 according to the instructions of the manufacturer (Invitrogen, Carlsbad, CA). Intracellular Env expression in transfected cells was detected by an indirect immunofluorescence assay in which the primary antibody was an MLV Env-specific monoclonal antibody (MAb), MAb 83A25 (9). Cells expressing the envelope protein were enumerated at 48 h after transfection, because this was when maximum expression of Env was detectable by Western blot analysis, as described below. Examination of 230 to 607 transfected cells by immunofluorescence microscopy in each of seven independent experiments indicated that average percentages of 25.6% and 21.1% of the transfected cells expressed the envelope proteins for MCF13 and NZB-9, respectively. Thus, the efficiencies of transfection were comparable for both *env* gene plasmids. Similar transfection efficiencies were also obtained when an expression plasmid for β -galactosidase was cotransfected as an additional control (data not shown).

To examine steady-state levels of the polyprotein precursor (gPr80^{env}) and cleaved surface (SU) forms of Env for each virus, we performed Western blot analysis of cellular extracts isolated from transfected cells. At 24, 48, and 72 h after transfection, we observed that the predominant form of the MCF13 envelope was the gPr80^{env} precursor, which was present at levels approximately 20- to 40-fold greater than that of SU (Fig. 1A, lanes 4, 7, and 10). In contrast, we detected nearly equivalent amounts of gPr80^{env} and SU for NZB-9, with a precursor to SU mean ratios that ranged from 1.9 at 24 h to 0.8 at 72 h posttransfection (Fig. 1A, lanes 5, 8, and 11). Analysis of the protein band intensities of all Western blots was performed with a Kodak EDAS 120 scanner and software. Slight

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FIG. 1. Analysis of MCF13 and NZB-9 MLV envelope proteins. (A) Western blot of protein extracts prepared at 24, 48, and 72 h after transfection of 1.6×10^6 mink epithelial cells with 25 μ l Lipofectamine 2000 (Invitrogen, Carlsbad, CA) and 25 μ g purified plasmid DNA of either empty pLNCX2 vector (P) or clones of MCF13 *env* (M) or NZB-9 *env* (N). The faint band detectable in the control (P) lanes resulted from nonspecific binding of primary MAb 83A25 (9). Protein extracts from mink cells productively infected with the MCF13 (C1) or NZB-9 (C2) virus were used as controls for migration of the envelope precursor (gPr80^{env}) and SU for each MLV, which are indicated with arrows. α -tubulin was detected as a loading control. (B) Pulse-chase analysis of envelope proteins in mink cells infected with MCF13 or NZB-9 MLV at a multiplicity of infection of 2.5 for 4 days. The cells were pulse-labeled for 30 min with [³⁵S]Met-Cys and chased for various times. For each time point, 200 μ g of protein extract was reacted with polyclonal goat anti-Rauscher murine leukemia virus gp70 serum kindly provided by S. Ruscetti (NCI, Frederick, MD). The immunoprecipitates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The lane labeled “serum” shows an immunoprecipitation control performed with normal goat serum.

differences in mobility between the MCF13 Env precursor and SU proteins and that of the corresponding NZB-9 glycoproteins were detectable, similar to what has been described for MCF247 MLV and the xenotropic 69X9 MLV (10). The results of pulse-chase analyses of envelope proteins for both viruses indicated that processing of the MCF13 polyprotein precursor occurred more slowly and incompletely than that of the NZB-9 polyprotein (Fig. 1B). For these assays, we used virus-infected cells because they contained greater amounts of envelope protein for immunoprecipitation.

Overexpression of the MCF13 MLV envelope protein induces cytotoxicity via apoptosis. To determine whether the envelope protein by itself can induce cell killing, we enumerated the cells that survived *env* expression by neomycin selection of transfected cells. Mink cells were transfected with plasmid DNA corresponding to either an empty vector or clones encoding MCF13 or NZB-9 Env and subsequently grown in medium containing 1,200 μ g per ml of G418. Colonies of neomycin-resistant cells growing on two or three plates for each *env* expression plasmid were enumerated at 19 to 21 days of growth in selectable medium in two independent experiments. As shown in Fig. 2, the colony count for cells that had been transfected with an empty vector was threefold greater than the number that survived transfection with MCF13 *env*-plasmid DNA ($P < 0.001$, as determined by the Student's *t* test). On the other hand, there was only a 1.4-fold difference between cells transfected with an empty vector and those infected with an NZB-9 *env*-plasmid DNA ($P = 0.003$). The number of cells that survived MCF13 *env* transfection was also lower by approximately twofold ($P = 0.001$) than that which survived transfection with NZB-9 *env*. These results thus indicated that expression of the MCF13 envelope protein by itself is able to induce cytopathic effects in mink epithelial cells and

that high levels of unintegrated viral DNA are not required for this effect.

To verify that the MCF13 MLV envelope protein can induce cell killing, we detected apoptotic cells after transfection of mink cells with the different plasmid DNAs. Staining with Hoechst 33342 dye was used to detect cells containing nuclei with altered morphology, a hallmark of apoptosis, by fluorescence microscopy. Apoptotic cells were enumerated at 24, 48, and 72 h after transfection (Fig. 3). Although no significant difference in apoptotic cell numbers between any of the plasmid DNAs (*P* values are between 0.2 and 0.5) was detectable at 24 h posttransfection, at 48 h posttransfection we did ob-

FIG. 2. Killing of mink epithelial cells by Env. Mink cells were transfected as described for Fig. 1 with plasmid DNA corresponding to either empty pLNCX2 vector, MCF13 *env*, or NZB-9 *env*. Forty-eight hours later, the cells were trypsinized, plated at dilutions of 20- and 40-fold onto two 10-cm plates for each dilution, and grown in selectable medium containing 1,200 μ g per ml of G418 for 15 to 19 days. Neomycin-resistant (neo^R) colonies were counted after being stained with crystal blue dye. The mean values and standard deviations of the numbers of colonies for mink cells transfected with the empty vector (black bar), MCF13 *env* (white bar), or NZB-9 *env* (striped bar) were calculated from the results of two independent experiments.

FIG. 3. MCF13 Env expression induces apoptosis in transfected cells. Mink epithelial cells were transfected as described for Fig. 1. Adherent and nonadherent cells were pooled from a 10-cm plate at 48 h after transfection, fixed with 1% paraformaldehyde and 0.1% Triton X-100 for 10 min, and stained with a solution of 1 μ g per ml of Hoechst 33342 dye (Molecular Probes, Eugene, OR) for 5 min. Nuclei were visualized with a Zeiss Axiophot fluorescence microscope and a Plan-NEOFLUAR 20X/0.5 objective utilizing a UV filter. Percentages of apoptotic cells are shown at the indicated times after transfection with plasmids containing either empty pLNCX2 vector (black bars), MCF13 *env* (white bars), or NZB-9 *env* (striped bars). Mean values and standard deviations were calculated from examining 380 to 1,200 transfected cells in each of two independent experiments.

serve significant differences between the percentages of apoptotic cells for MCF13 Env and those for the empty vector and NZB-9 Env. At this time point, the percentage of apoptotic cells induced by the MCF13 envelope was 6-fold greater than the percentage produced by the empty vector ($P < 0.001$) and 2.4-fold greater than that produced by NZB-9 Env ($P = 0.01$). Although the percentage of apoptotic cells induced by MCF13 Env declined at 72 h posttransfection, it was still significantly greater than that for either the empty vector or NZB-9 Env ($P = 0.01$ and 0.03 , respectively). At 48 h posttransfection but not at the other time points, we detected a twofold increase in the percentage of apoptotic cells for NZB-9 Env compared with that for the empty vector. This analysis demonstrated that cell killing by the MCF13 envelope protein occurs via apoptosis.

MLV envelope induces ER stress. Our previous studies demonstrated that MCF13 virus infection of mink cells resulted in the induction of ER stress (19). To determine whether the expression of MCF13 envelope protein alone induces apoptosis via this pathway, we analyzed cellular extracts from transfected cells for the upregulation of C/EBP homologous protein (CHOP) and glucose-regulated protein of 78 kDa (GRP78), which is diagnostic of ER stress (28). We performed Western blotting of protein extracts isolated from mink cells at 24, 48, and 72 h after transfection with either empty vector or plasmid clones expressing MCF13 or NZB-9 MLV envelope protein. We detected a significant upregulation of CHOP in cells transfected with the MCF13 *env* clone at 48 and 72 h posttransfection, when it was 3.6- and 3.9-fold greater, respectively, than in cells transfected with the empty vector (Fig. 4A). No comparable increase in CHOP was detectable in cells expressing NZB-9 Env at any time point. Upregulation of GRP78 also occurred at 48 h after transfection with the MCF13 *env*-plasmid, at which time it was 2.9-fold greater than in the control

FIG. 4. MCF13 Env upregulates CHOP and GRP78 in transfected cells. Mink epithelial cells were transfected as described for Fig. 1. Western blotting was performed with antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) to (A) CHOP or (B) GRP78 on protein extracts that were prepared at 24, 48, and 72 h after transfection with empty pLNCX2 vector (P), MCF13 *env* (M), or NZB-9 *env* (N). Cell extracts from mink cells either untreated (Un) or treated with 1 μ g per ml of tunicamycin for 18 h (Tm) were used as controls for ER stress. Increases (n -fold [fold-increase]) in intensity compared with that of the band in the respective control (P) lane, which was given the arbitrary value of 1, are indicated for the CHOP and GRP78 bands detectable for MCF13 (M) and NZB-9 (N). α -tubulin was detected as a loading control.

cells (Fig. 4B). No significant upregulation of GRP78 was detectable in NZB-9 *env*-transfected cells compared with that in the control cells. Our data thus indicate that MCF13 Env precursor polypeptide accumulation after transfection results in ER stress.

In this study, we demonstrated that the presence of the MCF13 envelope by itself can induce ER stress and apoptosis. Furthermore, we showed that these cellular effects correlate with the accumulation of the envelope precursor polypeptide after transfection. Notably, our data showed that the highest level of gPr80^{env} accumulation in transfected cells coincided with the time when peak levels of the ER stress-associated proteins CHOP and GRP78 appeared and the greatest percentage of apoptotic cells was detectable. These results taken together support the idea that ER stress induced by Env accumulation after MCF13 virus infection is the major pathway by which apoptosis occurs in mink epithelial cells. However, although our data indicate that high levels of unintegrated viral DNA are not required for apoptosis, it is possible that its presence may augment the degree of cell killing by the envelope protein.

Induction of ER stress by accumulation of the envelope precursor has also been detected for the neuropathogenic FrCas^E and Moloney ts1 MLVs, both of which are cytopathic for certain cell types (6, 7, 14, 15, 30). The mechanisms by which ER stress can progress to apoptosis when left unchecked most likely involve both mitochondrial-dependent and -independent pathways (3, 14, 28). However, the mechanism(s) by which a cell that is undergoing ER stress initiates the commitment to apoptosis is not well-understood. A number of studies have demonstrated that the envelope protein is an important determinant of pathogenicity and/or cytopathicity for patho-

genic retroviruses (8, 11, 15, 21, 23, 24, 26, 31). Although one obvious role for the MCF13 MLV envelope in T-cell lymphoma development is to function in receptor binding and entry into thymic lymphocytes, there may be additional roles for Env in disease progression that may contribute to tumorigenesis.

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