

Telomerase Activity in Chicken Embryo Fibroblast Cell Cultures Infected with Marek's Disease Virus

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Abstract

Background: Telomerase is a ribonucleoprotein, which adds telomeric repeats onto the 3' end of existing telomers at the end of chromosomes in eukaryotes. One hypothesis states that telomere length may function as a mitotic clock, therefore expression of telomerase activity in cancer cells may be a necessary and essential step for tumor development and progression.

Methods: The detectability of telomerase activity in chicken embryo fibroblast (CEF) cells infected with different passages of Marek's disease virus (MDV) was tested with the TRAPEZE[®] telomerase detection kit at passages 14 (P14), P80/1 and P120 for the Woodland strain, and passage 9 (P9) for the MPF57 strain.

Results: The results showed increased telomerase activity in MDV Woodlands strain at P14 and MPF57 strain at P9.

Conclusion: Our results suggest that MDV-transformed cells at low passage are a suitable system for the study of telomerases in tumor development and for testing telomerase-inhibiting drugs.

Keywords: Telomerase, Chicken embryo fibroblast, Marek's disease virus

Introduction

Telomeres are specific structures at the end of chromosomes in eukaryotes. In human chromosomes, telomeres consist of thousands of copies of six base (TTAGGG) repeats.¹⁻³ The telomerase is a ribonucleoprotein that synthesizes and

directs the telomeric repeats onto the 3' ends of existing telomeres using its RNA component as a template.³⁻⁶ Telomerase activity has been shown to be specifically expressed in immortal cells, cancer cells and germline tissues,⁴⁻⁷ where it compensates for telomere shortening

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during DNA replication and thus stabilizes telomere length. These observations have led to the hypothesis that telomere length may function as a "mitotic clock" to sense cell aging and eventually signal replicative senescence or programmed cell death.⁸ In addition, expression of telomerase activity in cancer cells may be a necessary and essential step for tumor development and progression.^{6,7} The development of a sensitive and efficient PCR-based telomerase activity detection method, the Telomeric Repeats Amplification Protocol (TRAP), has made it possible to survey telomerase activity in cells and tissues.^{9, 10}

Marek's disease (MD) is the most common lymphoproliferative disease of fowl and is characterized by mononuclear infiltration of peripheral nerves and other organs. It is caused by a herpesvirus, Marek's disease virus (MDV), and can be distinguished etiologically from other lymphoid neoplasms in birds. Replication of MDV is typical of other cell-associated herpes viruses with two recognized types of infections: productive, in which replication results in cell death, and non-productive or latent, in which there is little or no expression of viral genes and the infected cell remains viable. Cells affected with oncogenic serotype 1 MDV may also undergo neoplastic transformation.¹¹⁻¹³

Isolates of MDV have been obtained and used by many laboratories. These isolates differ principally in virulence and the tissue distribution of gross lesions. Three classes of viruses are capable of protecting chickens against MD: attenuated MDV, turkey herpes viruses and the naturally avirulent field strain of MDV. Woodlands no. 1 strain has been isolated from a flock of 14-week-old chickens at a meat breeder in Queensland, Australia. Attenuation of the virus has been observed at passage 60 or more in a chicken pathogenicity test. The MPF57 strain was isolated from a flock of 14-week-old Isa Brown layers on a farm located in Sydney, Australia. Both strains have been isolated from flocks with high levels of MD despite vaccination with attenuated live vaccines.¹¹

Induction of telomerase activity in a transformed avian lymphoblastoid cell line by MDV has been previously reported.¹⁴ The aim of this study was to test the detectability of telomerase activity in normal chicken embryo fibroblast (CEF) cells infected with different passages of Woodlands strain MDV, including passage 14 (P14), P80/1 and P120, and MPF57 strain P9, and uninfected and positive controls.

Materials and Methods

Infectivity assays

Duplicate day-old secondary CEF cultures were prepared with four 10^5 -cell/mL count in 96-microwell plates (Nunc, Denmark) for quantitative assay using the Karber method.¹⁵ Viruses were kindly provided by Dr. D. B. Delaney. Five days after inoculation, plates were examined for viral cytopathic effects (CPE). The results of infectivity assays at five days post-infection, for each virus, were as follows:

Woodlands P14 $TCID_{50}/mL=10^{2.6}$; Woodlands P80/1 $TCID_{50}/mL=10^{3.36}$; Woodlands P120 $TCID_{50}/mL=10^{4.36}$; and MPF57-Passage9 (P9) $TCID_{50}/mL=10^{3.55}$.

Inoculation of 100 TCID50 of each virus to cell cultures

Secondary CEF culture was prepared in 24-well plates (Nunc, Roskilde Denmark) for each virus along with controls. The equivalents of 100 TCID50 for each virus were made in minimal essential medium (MEM, Gibco BRL, Darmstadt, Germany) supplemented with 2% fetal calf serum penicillin (100 U/mL) and streptomycin (100 μ g/mL), and added to each well of 24-well plates, one plate for each of the viruses. Wells of one 24-well plate were considered as the control. The medium was changed one day post-inoculation and wells were observed for 6 consecutive days. Cytopathic effects appeared at day 5. Four wells of one of the vertical rows infected with one virus in the plates along with uninfected control wells were harvested on days 1 through 6 after infection.

Harvesting, washing, cell count and storage

For harvesting, cells in the wells were scraped, washed twice with PBS and counted. The equivalents of approximately 10^5 cells in the cell suspensions were poured into eppendorf tubes for each virus, along with controls. A final centrifugation at 8000 rpm for 5 min was done. After removal of supernatant PBS, cell pellets were stored at -80°C .

Extract preparation, protein estimation

Thawed cells were resuspended in 200 μl 1X CHAPS buffer and analyzed with the TRAPEZE[®] Telomerase detection kit (S7700, Chemicon, Millipore, Billerica, MA, USA) according to the manufacturer's instructions. These suspensions were incubated on ice for 30 min and then samples were centrifuged at $12,000 \times g$ for 20 min at 4°C (cold room). A total of 160 μl of supernatant was transferred into fresh Eppendorf tubes and protein content was estimated with the BIO RAD[®] estimation method.

PCR amplification (TRAP assay)

Enzyme activity induction and PCR amplification were done according to the TRAPEZE[®] kit manual using primers in the kit. PCR was run on a Perkin Elmer 2400, PCR machine in two steps of 94°C at 30 s and 59°C at 30 s for 33 cycles preceded by 30 min incubation of samples at 30°C to induce the telomerase reaction.

Polyacrylamide gel electrophoresis (PAGE) analysis

PAGE analysis was carried out on 12% polyacrylamide gel according to the TRAPEZE[®] kit manual.

Results

If telomerase was active in any extract it added a number of GGTTAG telomeric repeats onto the 3 end of substrate oligonucleotides, and the extended products were then amplified by PCR using the specific forward and reverse primers in the kit, generating a ladder of products with 6 base-

pair increments which started at nucleotides 50, 56, 62, 68, and so forth. Telomerase activity was detected in cells infected with Woodlands P14 and MPF57-P9 from day 5 post-infection, or when CPE was noted in CEF, where at least 50 and 56 telomerase nucleotide products were observed by PAGE analysis (Figure 1).

Figure 1 shows a representative gel. Telomerase products were detected to some extent by Woodlands low passage (P14) and MPF57-P9. No heat-treated PCR products including 50 base-pair nucleotides or the next heavier products with 6 base-pair increments were seen in related lanes (heat-treated controls), as the telomerase enzyme was inactivated by heating.

Discussion

The purpose of our work was to detect telomerase activity with different passages of MDV along with uninfected and positive controls. The results showed that telomerase activity was present in CEF cells 5 days post-infection with the Woodlands strain at P14 and the MPF57 strain at P9, which produced typical signs of MD. However, no telomerase activity was detected with higher passages of the Woodlands virus.

The presence of telomerase was detected in 98 of 100 cultured cell lines (transformed or cancerous cells) and 90 of 101 primary tumors. The fact that telomerase activity could not be

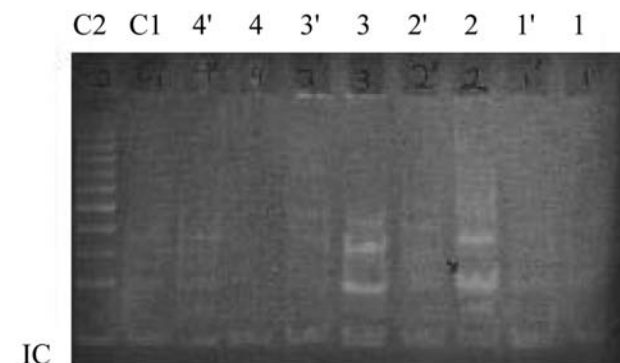


Figure 1. Pattern of telomerase product migration in extracts of controls and CEF cells infected with different passages of Marek's disease virus 5 days post-infection.

Key: 1, Woodlands P120; 2, Woodlands P14; 3, MPF57-P9; 4, Woodlands P120; C1, Negative control, 5-day-old CEF cells; C2, Positive control; IC, Internal control bands. Numbers with primes (e.g., 1, 2) are heat-treated controls.

detected in 22 normal somatic cell cultures and 50 normal or benign tissues provided strong support for the hypothesis that links telomerase activity to cell immortality.⁴⁻⁶

Marek's disease is an important model for the study of the tumorigenic potential of herpes viruses.^{12,13} The finding of increased telomerase activity in CEF cells infected with oncogenic MDVs, for the first time in this study, is consistent with a study reporting telomerase activity in an avian lymphoblastoid cell line transformed by MDV.¹⁴ Increased activity is also supported by data from other studies showing telomerase activity in Epstein-Barr virus- and cytomegalovirus-infected cells.^{6,8,16}

There are reports of increased telomerase activity by other tumor viruses such as human papilloma virus in cervical cancer,¹⁷ avian leucosis virus,¹⁸ and recently hepatitis B and C viruses.¹⁹ Proteins of the oncogenic viruses are known to affect telomerase expression and activity, frequently through multifunctional mechanisms.¹⁹ For MDV the probable mechanism is the deregulation of Bcl-2, or an induced mutation of tumor suppressor protein p53 in the infected cells, which can enhance telomerase activity.¹⁴

Lower pathogenicity has been observed at higher passages of MDV in CEF cells compared with lower passages and original field isolates.¹¹ It is tempting to speculate that the lower the passage of a tumor virus, the higher its potential to induce both tumors and telomerase activity, as in our study. It was also obvious from the results of infectivity assays that more infectious units (viral particles) are detectable in higher passages because of adaptation to growth. It has also been confirmed that tumor viruses do not replicate in transformed cells.²⁰

Interestingly, in species such as humans who survive for a number of years, repression of telomerase activity in somatic tissues evolved to reduce the probability of cancer.⁶ If this is true, some short-lived mammalian species might display weak repression of telomerase in somatic tissues, and thus a high frequency of spontaneous cell transformation, resulting in a high frequency

of cancer on a per-cell or per-year basis. Additional studies are needed to test this hypothesis. Our results have suggested that low passages of MDV-transformed cells are a suitable system for the study of telomerase activity and for the testing telomerase-inhibiting drugs.

Finally, according to this study, in vitro telomerase activity was not detected with less pathogenic MDV. Telomerase activity in infected cells, at least partly, would be a marker of tumor virus attenuation. There are some reports of a virus-encoded RNA telomerase subunit within the genome of oncogenic MDV strains but not in nononcogenic MDV strains.^{14, 21, 22} Thus, telomerase detection may be solely attributed to a tumor virus.

Recent data have shown that vaccination against MDV reduces telomerase activity in challenged chickens.²³ Telomerase expression in almost all advanced malignancies tested suggests that immortalized cells are required to maintain tumor growth. Further studies of telomerase biology either in cells or in tumor viruses such as MDV will potentially lead to more diagnostic and therapeutic applications.

Acknowledgments

We thank Dr. D. B. Delaney for providing MDV strains for this study.

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