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SHORT COMMUNICATION

The effect of age and telomere length on immune function in the horse

Madhu P. Katepalli, Amanda A. Adams, Teri L. Lear, David W. Horohov*

Maxwell H. Gluck Equine Research Center, Department of Veterinary Science, University of Kentucky, Lexington, KY 40546-0099, USA

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KEYWORDS
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Summary
Telomeres, specialized structures present at the ends of linear eukaryotic chromosomes, function to maintain chromosome stability and integrity. Telomeres shorten with each cell division eventually leading to replicative senescence, a process thought to be associated with age-related decline in immune function. We hypothesized that shortened PBMC telomere length is a factor contributing to immunosenescence of the aged horse. Telomere length was assessed in 19 horses ranging in age from 1 to 25 years. Mitogen-induced ^{3}H-thymidine incorporation, total serum IgG, and pro-inflammatory cytokine expression was also determined for each horse. Relative telomere length (RTL) was highly correlated with overall age. RTL was positively correlated with ^{3}H-thymidine incorporation and total IgG. Expression of pro-inflammatory cytokines was negatively correlated with RTL. These measures were also correlated with age, as expected. However, RTL was not correlated with immunosenescence and inflammaging in the oldest horse.

Introduction

Telomeres are specialized DNA and protein structures present at the ends of linear eukaryotic chromosomes that play an important role in maintaining the stability and integrity of chromosomes [1]. Since telomeres shorten with each cycle of cell division, telomere loss acts as a molecular 'clock' that triggers cell senescence [2]. While telomere length appears to control the progression of cell cycle, the actual molecular mechanisms responsible for this effect remain unknown. Replicative senescent cells express high levels of the cyclin-dependent kinase inhibitor p21 [3]. Since cyclin-dependent kinases are essential for the phosphorylation of retinoblastoma (pRb) protein, increased activation of p21 can prevent its phosphorylation [3]. Hypophosphorylated pRb actively represses several genes

Abbreviations: PBMC, peripheral blood mononuclear cells; RTL, relative telomere length; Flow-FISH, flow cytometry and fluorescent *in situ* hybridization; TROC, telomere length rate of change.

*Corresponding author. Tel.: +1 859 257 4757; fax: +1 859 323 3981.
E-mail address: DWHoro2@UKy.edu (D.W. Horohov).

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required for S phase by sequestering the E2F transcription factors, thus inhibiting cellular proliferation [4]. It has been proposed that p21 is activated by shortened telomeres [3].

Progressive telomere shortening with age in humans has been demonstrated in many studies [5–7]. It has also been observed that telomeres shorten with age in blood samples collected from other species [8] including non-human primates [9], birds [10] and donkeys [11]. A number of reports examining telomere length in peripheral blood mononuclear cells (PBMCs) have documented a correlation between shortened telomeres and a wide variety of aging-related diseases [1,12–15]. A correlation between shortened PBMC telomeres and increased risk for death has even been found in subgroups of patients [16]. While the underlying mechanism relating telomere length with age-related diseases is unclear, there is evidence to suggest that alterations in immune function play a role [1,17].

Rapid proliferation and clonal expansion of a number of antigen-specific naïve and memory lymphocytes is essential for the effective functionality of the immune system [18]. Any limitation on the process of cell division could have devastating effects on overall immune function. Therefore, telomere-driven replicative exhaustion has been proposed as a mechanism leading to immunosenescence in both T and B lymphocytes [19,20]. Age-associated loss of telomeres has been observed in different types of leukocytes [21–23]. While both lymphocytes and granulocytes exhibit telomere attrition with age, lymphocytes show a greater degree of shortening than granulocytes (59 bp/year versus 39 bp/year, respectively) [24]. Both naive (CD27−) and memory (CD27+) B cells lose telomere repeats with age, there is no consistent difference in telomere length between these two B cell subsets [25]. Within the T cell compartment, where telomere shortening occurs in both CD4+ and CD8+ T cells [24,26,27], memory T cells in both compartments have shorter telomeres than their naive counterparts, suggesting that cellular proliferation is the primary stimulus for telomeric attrition [28]. While T cells also increase telomerase activity when activated, this process is tightly regulated and eventually lost in stimulated cells [28]. Failure to regulate this process may be associated with pathologic conditions [29].

Based on these observations, it is clear that telomere erosion has direct implications for the immune system. Given the significant impact of telomere-driven replicative senescence in the age-related changes of the immune system in humans and many other species studied so far, we hypothesize that PBMC telomere length is a factor contributing to the immunosenescence of the aged horse. Here, we compare telomere length in the peripheral blood mononuclear cells of young and old horses to determine the correlations between age, telomere length and various parameters of immune function.

Materials and methods

Horses

A total of 19 horses of mixed breeds ranging in age from 1 to 25 years were included in this study. The horses were part of a larger research herd maintained by the Department of Veterinary Science, University of Kentucky at the Maine Chance Farm located near Lexington, Kentucky. All horses were maintained on pasture with *ad libitum* access to water and forage and handled in accordance with the Guide for the Care and Use of Agricultural Animals in Agricultural Research. All research procedures were approved by the University of Kentucky's Institutional Animal Care and Use Committee.

Peripheral blood mononuclear cells

Fresh peripheral blood samples were collected into 15 ml heparinized tubes (BD Vacutainer, NJ) by jugular venipuncture. The tubes were allowed to settle for 20 min to form a leukocyte-rich plasma layer. Peripheral blood mononuclear cells (PBMCs) were then isolated using density gradient centrifugation. The PBMCs were re-suspended in 10 ml of complete (c) RPMI 1640 (Gibco, Grand Island, NY), supplemented with 2.5% fetal equine serum (Sigma Aldrich, St. Louis, MO), 2 mM L-glutamine (Sigma), 100 U/ml penicillin, streptomycin (Sigma) and 55 μM 2-mercaptoethanol (Gibco) and prepared for counting by diluting 100 μl of cell suspension into 900 μl of PBS. The cells were counted using a cell counter (Vi-cell Counter XR, Beckman Coulter, Miami, FL), which determined both total cell numbers and viability.

Flow cytometry and fluorescent in situ hybridization (Flow-FISH)

Telomere length in PBMCs was determined using a fluorescence-based *in situ* hybridization kit (Dako Cytomation, Glostrup, Denmark) and flow cytometric analysis [30]. This method entails the *in situ* hybridization of a fluorescein-conjugated peptide nucleic acid (PNA) telomere probe to chromosomal DNA in solution. Telomere length was determined as relative telomere length (RTL) by comparison with an internal cell line control (CEM-1301-2W, Dako). Samples were acquired using the FACS Calibur (Becton Dickinson, San Jose, CA) and the data were analyzed using Cell Quest software version 3.1. A logarithmic-scale FL1-height was used for probe fluorescence and linear scale FL3-height for DNA staining. A gate was set on the $G_{0/1}$ phase for both the PBMCs and control cells so that diploid only cells were analyzed and the estimated RTL is per genome equivalent. Telomere fluorescence was calculated by subtracting the mean fluorescence of the background control (without probe) from the mean fluorescence obtained from cells hybridized with the telomere probe in order to determine RTL as

$$\frac{\text{Mean FL1 PBMCs (with probe} - \text{without probe)} \times \text{DNA index of control cells} \times 100}{\text{Mean FL1 control cells (with probe} - \text{without probe)} \times \text{DNA index of PBMCs}}$$