Int Arch Occup Environ Health (1993) 65:S235–S240

International Archives of
Occupational and Environmental Health

Retroviral gene expression as a possible biomarker of aging

Yasuhiko Wada[1], Mikako Tsukada[2], Sigetosi Kamiyama[2], and Akio Koizumi[2]

[1] Akita Prefectural Oodate Health Center, Oodate 017, Japan
[2] Dept. Hygiene, Akita University School of Medicine, Akita 010, Japan

Summary. We examined effects of aging on endogenous retrovirus gene expression of mouse lymphocytes with a hypothesis that it may be a useful biomarker of aging. Mice have endogenous murine leukemia viruses (MuLVs) in their chromosomes. We detected the gene expression of long terminal repeats (LTRs) of MuLVs. Brains, livers and spleens were taken from young (3 months old) and old (27 months old) male C57BL/6 mice. In addition to these control (C) mice, we also determined gene expression in dietary restricted (DR) mice, in which rates of aging are known to be slowed. RNA was extracted from the tissues and converted into cDNA. The MuLV-LTR portion of cDNA was amplified by polymerase chain reaction (PCR). The PCR products were analyzed by agarose gel electrophoresis. Gene expressions of young mice were found to be tissue-specific. Expressed LTRs from brains, livers and spleens were that of 370 base-pairs (bp), those of 370 and 620 bp, and those of 370, 400 and 620 bp, respectively. Old mice of C group, however, decreased tissue specificity: expressed LTRs became those of 370-400 bp in any tissues. In contrast the tissue specific gene expression was conserved in old DR mice which had to get prolonged life span and decreased lymphoma incidence. Thereby, gene expression of endogenous retroviruses appears to change during aging and to be modifiable by life-prolonging DR. It may be therefore used as a biomaker of aging in mice. Humans are known to have similar gene elements like MuLV. The present findings demonstrate a possibility of application of endogenous gene expressions to the epidemiology of aging.

Key words: Biomarker – Dietary restriction – Murine leukemia virus – PCR – Rate of aging

Introduction

The research for biological markers of aging has been the focus of gerontologic research for many decades. The goals of identifying boimarkers of aging (Mooradian 1990) are; 1) understanding the various determinants of aging; 2) monitoring the impact of various interventions on the rate of aging; 3) determination of biological age of the individual; and 4) estimation of life expectancy.

At present, the only way to monitor the rate of aging is to rely on retrospective observations. The availability of a biomarker of aging will allow determination of the rate of aging within limited time periods and therefore will allow monitoring of the impact of various interventions, such as exercise, nutritional manipulation, on the aging process.

In the mammalian genomes, there are many endogenous retroviruses which take the form of proviruses. The typical examples of these proviruses are MuLV and mouse mammary tumor virus (Koizumi et al. 1990). These proviruses are not actively transcribed when hosts are young but are expressed during aging. Once the retroviruses are expressed, they behave like ordinary retroviruses. They are infectious and are incorporated into the genome to result in insertional mutagenesis of the gene (Lowy 1985). Mutations of viral sequences occur very often because of the infidelity of enzymes involved in transcription such as reverse transcriptase and viral polymerases, which do not carry editing functions. The mutations thus are assumed to accumulate during aging (Lowy 1985). The first aim of the present study, therefore, was to examine the effects of aging on endogenous gene expression of MuLV in the lymphocyte with a hypothesis that it may be a useful biomarker of aging.

A biomarker of aging should be modulated by the same factors that are known to alter the rate of aging. Dietary restriction (DR) without malnutrition decelerates aging processes and should retard the emergence of the putative biomarker of aging (Koizumi et al. 1990). Thus the second aim of the present study was to examine the effects of DR on gene expression of murine leukemia viruses.

Materials and methods

Mice. C57BL/6 (B6), 4 weeks of age, were purchased from Nippon Clea Tokyo, Japan). Mice were assigned randomly to either a control (C) or a DR group upon arrival at our vivarium or at the time of weaning. The animals were individually housed in plastic cages with wood shavings at 20-22°C, with a relative humidity of 50% and a 14-h light (0400-1800)/10-h dark (1800-0400) photocycle. Mice were killed by cervical dislocation at ages of 3 ($n = 3$) and 27 ($n = 3$) months. Brain,

liver, and spleen (also tumor tissues when found) were collected and stored in liquid nitrogen until use. Other animals (C: n = 38, DR: n = 32) were kept until their natural deaths. The mice were maintained under pathogen-free conditions throughout the experiment. Body weights were determined every four weeks. All animals were handled in accordance with the animal welfare guidelines of Akita University.

Diet. The formulations of the two diets in the present study were previously described (Koizumi et al. 1990). Two diet groups were studied. 1) Control: Mice in this group ate the control diet, a purified, pelleted, 23.2% casein diet at a level of 397 kJ/wk. The control mice were given 27 g per week which averages 20% less than the amount consumed by mice given completely free access to the diet. 2) DR: Mice in this group ate 201 kJ/wk of the restricted diet, a purified, pelleted, 39.7% casein diet further enriched with vitamin and mineral content. The mice were given 16 g per week, thereby consuming approximately the same amount of protein, fat, vitamins and minerals per week as did the control mice, but only 32 % as much carbohydrate. The control B6 mice were fed 4 g of control diet daily on Monday through Thursday morning and 11 g on Friday morning. The DR mice were fed 3.5 g of the DR diet per day on Monday and Wednesday and 9 g on Friday mornings between 0900 and 1000 as previously reported (Koizumi et al. 1990).

DNA isolation from tissues. Tissues were homogenized in homogenizing buffer (0.1 M NaCl, 0.2 M sucrose, 0.01 M EDTA, 0.3 M Tris-HCl, pH 8.0), denatured by sodium dodecyl sulfate and digested with excess amounts (400 Units/ml) of proteinase (Wako Pure Chemicals, Osaka, Japan) at 65°C overnight. Tissue samples were centrifuged and the supernatant fraction was collected. DNA was extracted from the supernatant fractions of brain, liver and spleen (and tumor when found) as described by Maniatis et al. (1982), ethanol precipitated and stored at -20°C until used for polymerase chain reaction (described below).

Isolation of total RNA from tissue samples. Total RNA was isolated from brain, liver, and spleen (and tumor when found) using Total RNA Separator (CLONTECH, Palo Alto, CA, USA) based on the acid guanidium method (Chomczynski et al. 1987). In brief, tissue samples (0.02 - 0.08 g) were homogenized in 0.8 ml of denaturing solution (4 M guanidium thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5 % sarcosyl and 0.1 M ß-mercaptoethanol) and RNA was extracted from the denaturing solution with 2M sodium acetate (pH 4.0) (80 µl), water-saturated phenol (0.8 ml) and chloroform-isoamyl alcohol (49:1) mixture (160 µl). RNA was precipitated with isopropanol at -20°C for 1 hr. The RNA pellet was freeze-dried and was dissolved in denaturing solution. The extraction procedure was repeated twice. The RNA was ethanol precipitated, and stored (-80°C) in TE buffer (10 mM Tris, 1 mM EDTA, pH 7.5) until used. Concentrations were determined by absorbance at 260 nm.

Reverse transcription of total RNA. The RNA solution was dissolved in the reverse transcription reaction mixture as described below. Total RNA was reverse transcribed by addition of 500 pM random hexamer (Takara, Tokyo, Japan), 200 µM deoxynucleotides, 1 × reaction buffer (10 mM Tris-HCl, pH 8.3, KCl 50 mM, $MgCl_2$ 1.5 mM, gelatin 0.001% (W/V), ribonuclease inhibitor 20 units, and dithiothreitol 0.4 mM), and 400 U of Moloney murine leukemia virus reverse transcriptase (M-MLV TR) (United States Biochemical, Cleveland, USA) in a 50 µl. The reaction was conducted at 23°C for 10 min, at 37°C for 60 min and at 94°C for 5 min followed by immediate cooling at ice temperature.

Amplification by polymerase chain reaction (PCR). A pair of forward (5') and reverse (3') primers for endogenous murine leukemia provirus examined in the present study are shown in Figure 1 (Rassart et al. 1986). The mixture contained 25 ng of DNA or cDNA corresponding 2.5 µg RNA in reaction buffer (Tris-HCl 10 mM, pH 8.3, KCl 50 mM, $MgCl_2$ 1.5 mM, gelatin 0.001% (W/V)), 0.03 µM of 5' and 3' primers, 200 µM deoxynucleotides and 1.25 U of AmpliTaq DNA polymerase (Takara, Tokyo, Japan) in 50 µl. The PCR profile was as follows: 94°C for 1.5 min to melt the DNA, 58°C for 2 min to allow primer annealing and 72°C for 2 min for DNA extension. The PCR profile was repeated for 30 cycles.

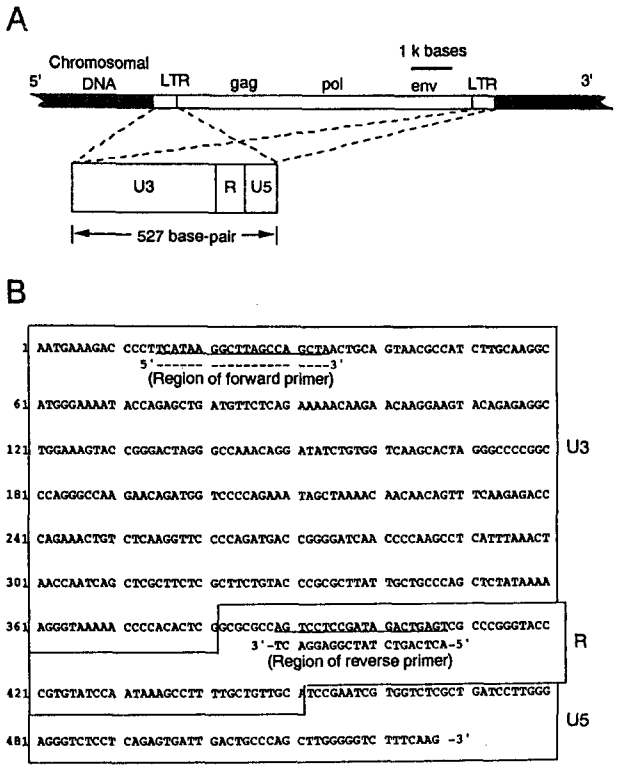

Fig. 1. The structure of the ecotropic murine luekemia provirus (A) and DNA sequence of the LTR region of C57BL mice (B).

Detection of PCR products. Primers were designed to amplify a 394 base-pair DNA fragment of the ecotropic MuLV's LTR (Rassart et al. 1986) and other LTRs which had homology to it, and identification of products was achieved by comparison of amplified products with molecular weight standard: Ø X DNA cleaved with Hae III. Products were detected by agarose gel electrophoresis. Samples (two fifth of the PCR products) were electrophoresed on an agarose gel (2%) at 110V/25 cm for 70 min. Gels were stained with 0.5 µg/ml ethidium bromide for 30 min. When indicated, the agarose gel was Southern blotted and hybridized with ^{32}P-MuLV genome as described below.

Southern blotting. Southern blotting was conducted as described by Maniatis et al. (1982). The DNA samples of PCR products were electrophoresed in a 2% agarose gel and the gel was transferred to a nylon membrane. Abelson murine leukemia virus genome inserted in plasmid pBR322 was obtained from Japanese Cancer Research Resources Bank-Gene (Tokyo-Japan). The MuLV DNA fragment was ^{32}P labeled by a random primer kit (Takara, Tokyo, Japan) to a specific activity of 1-5 × 10^7 cpm/µg as described by Maniatis et al. (1982). The nylon membrane was hybridized and washed as described by a supplier of the

nylon membrane (Du Pont).

Histopathological examination of tumors. A part of brain, lung, liver, spleen, kidney and tumors (if found) were fixed in 10% formalin, were sectioned into 10 μm thick samples and were stained with hematoxylin and eosin.

Statistics. Differences in tumor incidences or mortality were analyzed by Fisher's exact test (Gad and Weil 1989). Survival rates and onsets of lymphoma deaths in DR and control mice were compared using Cox-Mantel test (Gad and Weil 1989). A value of $P < 0.05$ was considered significant.

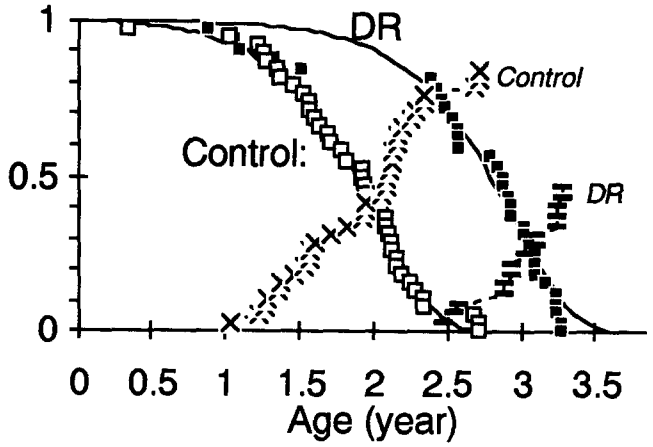

Fig. 2. Survival curves and incidence of lymphoma death. Open squares: the survival curve of control. Closed squares: the survival curve of DR mice. Curve lines are Gompertz's curves. X: a death of lymphoma in control. A bar: a death of lymphoma in DR mice.

Results

Survival curves, mortalities, growth curves, and tumor incidences

The ratio of the mean body weight of the DR mice to that of the control mice was 0.50 (40 g *vs.* 20 g), roughly equal to the ratio of the energy intakes.

Survival curves and cumulative lymphoma incidences are summarized in Fig. 2. DR increased a survival rate significantly ($P < 0.001$), and delayed the onset of lymphoma. It also reduced net incidences of lymphoma significantly (p = 0.001) (Table 1). The earliest lymphoma death in control mice occurred at 12 months of age and 50% of lymphoma deaths had occurred by 25 months of age. In contrast the earliest lymphoma deaths occurred in DR mice at 30 months of age and 50% of lymphoma deaths occurred by 35.5 months. The increased life span in DR mice appears to be mainly the result of a lower incidence of neoplastic diseases.

Confirmation of PCR products as MuLV-related gene products

The major PCR products of genomic DNA in three organs contained two molecular species, 400 and 620 bp products as shown in Fig. 3A (the right, upper portion). Other minor products contained 370, 440, 500 and 680 bp products. Southern blotting were executed using an Abelson MuLV DNA as a probe to confirm that these products were MuLV-related DNA fragments. The major and minor products hybridized with the ^{32}P-labelled Abelson MuLV DNA (data not shown), suggesting that these PCR products contained LTR portions of MuLV. The sequence homology search with GenBank Database suggested that the 400 bp product corresponded to the endogenous ecotropic proviral LTR (an expected product size is 394 bp) (Rassart et al. 1986) and 620 bp product corresponded to MuLV-related (modified polytropic) provirus LTR (an expected size was 611 bp) (Stoye and Cofan 1987).

Table 1. Tumor incidences, longevities and maximum life spans of C57BL/6 male mice.

Group	Neoplastic diseases: Lymphoma	Neoplastic diseases: Lung tumor (No. of mice)	Non-neoplastic diseases[a] (No. of mice)	Age of 50% survival[b] (Years)	Age of the last death[b] (Years)	Comparison of survival rate[c]
Control (n=38)	32*	1	5	1.89	2.71	Z=6.17
DR (n=32)	15	0	17	2.80	3.25	P<0.001

[a] Non-neoplastic diseases include unknown causes of death, hydronephrosis, and bleeding.
[b] Age of 50% survival indicates the age when 50% of mice survived (i.e., died). Age of the last death indicates the age when the last mice died.
[c] Survival rates of control and DR mice were compared using Cox-Mantel test (See Materials and methods).
* indicates that control mice had higher incidences than DR mice ($P < 0.05$) by Fisher's exact test.

Variability of LTR portion of proviral MuLV DNA during aging in control and DR mice

We analyzed the PCR products of genomic DNA samples from brain, liver, spleen and tumors. Products of 400 and 620 bp fragments were consistently found in all organs including tumors from control and DR mice at 3 and 27 months of ages (data not shown). The present result indicates that a large scale deletion more than 10 bp did not occur in LTR portion of MuLV proviruses during aging.

Tissue- and age-specific gene expression of MuLV LTR portion in brain, liver, spleen and tumors during aging in control and DR mice

To confirm that the PCR products were derived from cDNA, not directly from contaminated DNA, we executed PCR using total RNA fraction without reverse transcriptase. The PCR products were not produced in experiments when the reverse transcriptase was omitted (data not shown). This evidence suggested that the PCR products were derived from RNA, not from contaminated trace amount of provirus DNA.

We first examined patterns of LTR RNAs in various organs from control (n = 3) mice of 3 months of age. As shown in Fig. 3A (the upper portion), brain, liver and spleen had three different RNA species, which were characteristic to individual organs. A single RNA (370 bp)

was found in the brain, and two products (370 and 620 bp) were observed in the liver while three products, 370 bp, heterogeneous products around 400 bp and a product of 620 bp, were detected in the spleen. In DR mice, the same patterns of the tissue specific gene expression were found.

Those tissue specific gene expressions were, however, disappeared in normal tissues from control mice (n = 3) at 27 months of age (Fig. 3B, the top half). The products of smaller LTR fragments, 370 bp or 400 bp products, were found in brain, liver and spleen, but the larger (620 bp) product almost disappeared in the liver and spleen. In contrast, RNA from lymphomas maintained a similar pattern to that of liver and/or spleen of 3 months old mice.

We next examined the effects of DR on age-related alterations of tissue specific gene expression in the same tissues at 27 months (Fig. 3B, the bottom half). The 620 bp product was still detected in liver (from 3/3 mice) and spleen (from 2/3 mice) even at 27 months of age. In the spleen, the heterogeneous products of which molecular sizes are about 400 bp were still found at 27 months of age. Those findings indicate conservation of tissue specificity of gene expression in DR mice at old ages.

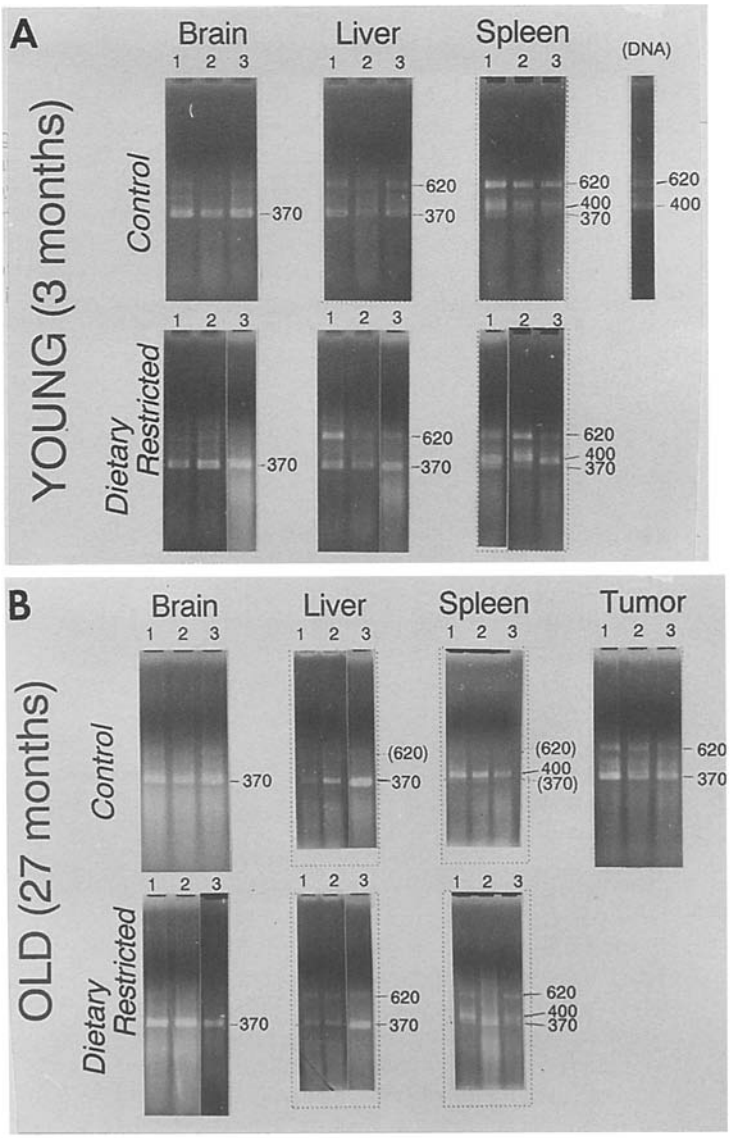

Fig. 3. Tissue-specific retroviral gene expression disappeared in old control mice, but was conserved in old DR mice.

Discussion

To our best knowledge, this study is the first report on the effects of aging and DR on the gene expression of MuLV in mice. We detected at least six MuLV provirus loci and their gene products. The relative abundance of the gene products is assumed to represent to what extent the individual gene loci of MuLV proviruses are expressed. We found tissue specific gene expression of MuLV proviruses: a single mRNA species was found in the brain: two mRNA species were found in the liver: three mRNA were found in the spleen. This tissue specific gene expression was obscured in these three organs by aging. The tissue specific gene expression was, however, maintained in the brains, livers and spleens of mice on DR during aging. It was also maintained in lymphoma. We thus speculate that DR reserves stem cells capable of proliferation and these cells conserved the gene expression patterns in young ages.

As shown above, the gene expression of MuLVs showed a qualitative changes during aging. It occurred in normal tissues such as brain, liver and spleen simultaneously. Since it was the same in organs of control and DR mice when they are young, the age-related alteration of tissue specific gene expression of MuLVs is not secondary to metabolic or nutritional changes. Finally, dietary restriction, which is known to delay aging rates, maintained the pattern in young ages. We conclude that the tissue specific gene expression of MuLV proviruses meets criteria of biomarkers (Mooradian 1990) and may be useful as a biomarker of aging in mice. Its advantage over other methods is that its information can be obtained by using peripheral white blood cells. It remains unknown at present whether the retroviral gene expression may be a biomarker in humans, which are known to have provirus loci similar to MuLVs in the genomes (Lowy 1985). More work needs to be done to elucidate these points.

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