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TELOMERASE ACTIVITY IN THYMOMAS AND MAMMARY GLAND ADENOCARCINOMAS INDUCED BY POLYOMA VIRUS IN AKR MICE*

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Abstract Telomerase is an enzyme that stabilizes telomere length in transformed cells and tumors. Its role in tumor development is far from clear. In this paper, a new experimental model to study telomerase activity during tumorigenesis is presented. After infection with Polyoma virus, AKR mice developed thymomas and mammary gland adenocarcinomas. Polyoma antigens were observed by the peroxidase-antiperoxidase technique on tissue sections, and by Western blot on tumor extracts. The TRAP assay was performed to detect telomerase activity. It was not present in normal mammary gland, but it was positive in mammary gland adenocarcinomas. A different pattern was seen in thymic tissues: normal thymus had higher telomerase activity than thymomas. The incubation of thymoma extracts with normal thymus extracts decreased telomerase activity in the latter. These results demonstrate two different patterns of telomerase activity in tumors induced by Polyoma virus, and suggest the presence of telomerase inhibitory factors in thymomas.

Resumen ***Actividad telomerasa en timomas y adenocarcinomas de mama inducidos por el virus Polioma en ratones AKR.*** La telomerasa es una enzima que estabiliza la longitud de los telómeros en células transformadas y en tumores. Su función en el desarrollo neoplásico no es clara. En este trabajo se presenta un nuevo modelo experimental para estudiar la actividad telomerasa durante la tumorigénesis. Se inocularon ratones AKR con virus Polioma, los cuales desarrollaron timomas y adenocarcinomas de mama. Los antígenos de Polioma fueron detectados en cortes histológicos por peroxidasa-antiperoxidasa, y en extractos tumorales por Western blot. Para estudiar la actividad telomerasa se empleó el ensayo TRAP. No se detectó actividad telomerasa en tejido mamario normal, pero fue positiva en adenocarcinomas de mama. Por el contrario, el timo normal tuvo mayor actividad telomerasa que los timomas. La incubación de extractos de timomas con extractos de timo normal produjo una disminución de la actividad telomerasa en estos últimos. Estos resultados demuestran dos tipos distintos de actividad telomerasa en tumores producidos por virus Polioma, y sugieren la existencia de inhibidores de la telomerasa en los timomas.

Key words: telomerase, Polyoma, TRAP assay, thymoma, mammary gland adenocarcinoma

Telomeres are structures located at the ends of eukaryotic chromosomes. They are composed of short repeated DNA sequences rich in G residues on one strand. Humans, mice and other mammals, contain TTAGGG repeats arranged in tandem, running 5' to 3'. The number of repeats varies in different species: for example, telomeres of the laboratory mouse *Mus musculus* are larger than the human telomeres.

It is acknowledged that telomeres both stabilize chromosome ends and are involved in the regulation of cell replication timing, chromosome positioning in the nucleus, and other important functions that control cell division[1].

In every normal somatic cell division, 50-200 nucleotides of telomeric sequence become lost[2]. It has been argued that the progressive shortening of telomeres is a "mitotic clock" that finally conduces normal cells to replicative senescence[3]. Immortal cells, however, do not present any reduction of telomeric DNA tandem repeats. This strongly indicates that the maintenance of telomere length is crucial for the cell to elude senescence and proliferate[4]. Telomeres are seemingly directly involved in cellular aging and in the pathogenesis of cancer.

Telomerase is an enzyme that either maintains or increases the length of telomeres. It is a ribonucleoprotein in which the RNA component is a template for the synthesis of telomeric DNA repeats onto chromosomal ends[1]. Telomerase activity is detected in most tumors and stem cells, but is usually absent in normal tissues[5]. It can therefore be infered that telomerase may play some

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role in tumor production, and is currently considered a target of anti-tumoral therapy.

Several experimental approaches have been based on this hypothesis. For example, *in vitro* transformation of human B lymphocytes by Epstein-Barr virus has been associated with telomerase activity[6], mouse mammary tumor virus long terminal repeat-driven Wnt-1 proto-oncogene expression in mammary tissue of transgenic mice produces mammary tumors with high levels of telomerase[7], and HPV-16 E6 transduction of human keratinocytes and mammary epithelial cells activates telomerase[8].

Nevertheless, *in vitro* cell transformation does not correlate necessarily with the *in vivo* mechanisms of tumor production. The construction of transgenic mice or the transduction of cells with viral oncogenes can provide pertinent information on the relationship between oncogene expression and telomerase activity, but these systems are ultimately artificial.

The purpose of this paper is to present a new experimental model, in which telomerase activity is studied in tumors induced by Polyoma virus. The use of an infecting oncogenic virus permits highly reproducible results. Moreover, Polyoma is a natural virus of mice, and it has the property of infecting and of producing tumors in the same animal[9, 10], thus enabling a step by step study of tumorigenesis.

The PTA strain of Polyoma virus was used in every experiment. This strain, highly tumorigenic in mice, was obtained as a kind gift from Dr. Thomas L. Benjamin (Harvard Medical School, Boston, U.S.A.). Virus stocks were produced in primary cultures of specific-pathogen-free Balb/c mice kidney epithelial cells (BMK). When cytopathic effect was completed, cultures were frozen and thawed 3 times and centrifuged at 400 g during 20 minutes. The supernatant was titrated by the plaque-forming units method (pfu)[11]. Virus stock was aliquoted, and maintained at -70°C until used.

Newborn (less than 48 hs of life) AKR mice obtained from the mouse colony of the Academia Nacional de Medicina, Buenos Aires, were injected subcutaneously with 10^5 pfu of virus. Other mice were injected with the supernatants of uninfected BMK cells, and a third group was kept without any inoculation to evaluate the eventual incidence of spontaneous tumors. Each experimental group was composed of 20 animals, maintained 5 to a box, and fed on pellets *ad libitum*. Twice a week mice were observed clinically for the purpose of detecting tumors.

Between 60 and 90 days pi, 20/20 mice infected with PTA showed progressive dyspnea, and were sacrificed with an excess of ether anesthesia. In all 20 mice, necropsies showed the presence of tumors in the superior mediastinum. Tumors were large, soft and pink, occupying most of the thoracic cavity. Two of the mice also had subcutaneous nodules. Neither mock-infected nor untreated mice developed any tumors.

Tissues were immediately fixed in Bouin's fluid, and embedded in paraffin. Hematoxilin-Eosin, Gomori's silver impregnation, Masson's trichromic, and P.A. Schiff stains were used for histologic tumor characterization.

Mediastinal tumors were thymomas, and subcutaneous neoplasms were well differentiated intraductal mammary gland adenocarcinomas (Fig. 1). Polyoma structural antigens were detected in all tumors using, on tissue sections, the peroxidase-antiperoxidase (PAP) technique counterstained with Hematoxilin. The primary antiserum was a polyclonal one against Polyoma, prepared in rabbit. Intense positive labeling was observed in the nuclei of scattered tumor cells. Thymic tissue of uninfected mice was used as negative control for PAP technique, and tumors treated with normal rabbit serum as primary antibody were employed for internal control. Polyoma antigens were not detected in any control. The presence of Polyoma antigens in tumors was also comfirmed by SDS-Polyacrylamide Gel Electrophoresis and Western blot (Fig. 1).

Immediately after the mice were sacrificed, suitable samples of tumor and of normal tissues were taken and frozen in liquid nitrogen, then kept at -70 °C until telomerase detection was performed. Telomerase activity was studied by the TRAP assay, developed by Kim *et al*[5], and modified by Blasco *et al*[12]. Briefly, tissue were thawed, washed with Ice-Cold-Wash Buffer (10 mM HEPES-KOH pH 7.5; 1.5 mM Mg_2Cl; 10 mM KCl; 1 mM dithiothreitol), then Dounce homogeneized with Ice-Cold-Lysis Buffer (10 mM Tris-HCl pH 7.5; 1 mM Mg_2Cl; 1 mM EGTA; 5 mM β-mercaptoethanol; 0.1 mM phenylmethylsulfonyl fluoride; 0.5% w/v CHAPS; 10% v/v glycerol) and incubated on ice for 30 minutes. The cellular extract obtained was centrifuged at 16 000 Xg, 30 minutes at 4°C, and supernatants were aliquoted and kept at -70°C. Protein concentration was determined by the Bradford method (Biorad). An extension reaction was done using 3 different total protein concentrations (1 µg, 0.1 µg and 0.01 µg) of each sample to measure telomerase activity. The final concentration of the components in the telomerase extention reaction was: 50 mM Tris-Acetate pH 8.5; 1 mM spermidine; 5 mM β-mercaptoethanol; 3 mM Mg_2Cl; 50 mM potassium acetate; 1 mM EGTA, 0.5 µg of TS oligonucleotide (5' AATCCGTCGAGCAGAGTT3'); 2 mM each of dTTP, dGTP and dATP. Every reaction was done in a final volume of 40 µl and incubated 30 minutes at 30°C. Extracts of testes (where telomerase activity is normally present in mice) were used for telomerase extension as positive control. Since telomerase has a RNA component, RNAse and heating at 70°C 10 minutes were employed on the testes extract for internal control in every experiment. After the extension reac-

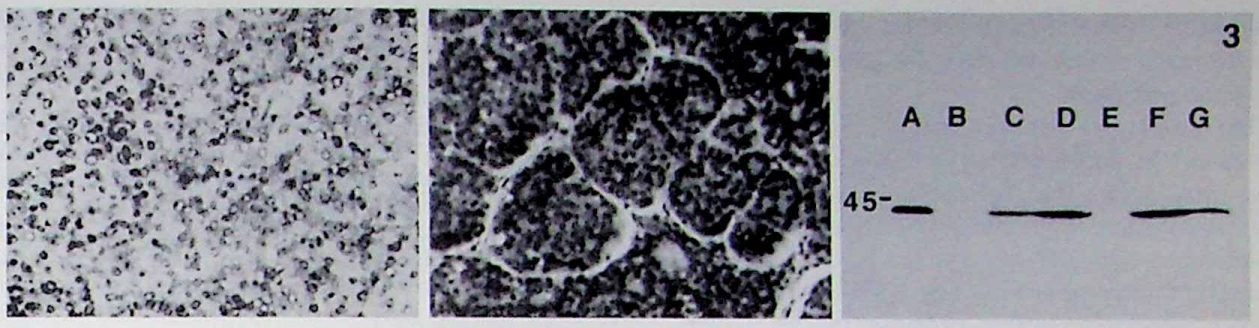

Fig. 1.– Histology of thymoma[1], mammary gland adenocarcinoma[2], and Western blot to detect Polyoma major capsid protein VP-1 in these tumors, developed by chemoluminiscence[3] A: purified VP-1, B: normal thymus, C-D: thymomas, E: normal mammary gland, F.G: mammary gland adenocarcinomas.

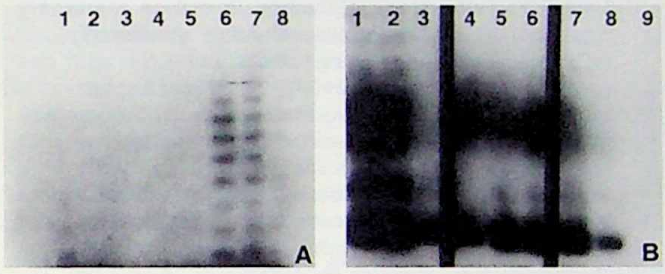

Fig.2.– Telomerase activity. A. 1: positive control, 2: positive control treated with RNAse and heat, 3-5: normal mammary gland extract 6-8 mammary gland adenocarcinoma extract. From left to right, lines 3 to 8: 1 µg, 0.1 µg and 0.01 µg of protein in each sample. B. 1-3: normal thymus extract, 4-6: thymoma extract, 7-9: thymoma extract plus normal thymus extract. From left to right 1 µg, 0.1 µg and 0.01 µg of protein in each sample.

tion, a PCR amplification of every sample was done using the TS primer already described, and the CX primer designed as a telomeric sequence (3' AATCCCATTCCCATTCCCCATTCCC5'). The reaction was composed of 10 µg of the extension product in 50 µl of TRAP buffer (20 mM Tris-HCl pH 8.3; 1.5 mM Mg_2Cl; 63 mM KCl; 0.005% Tween 20; 1 mM EGTA; 50 µM of all four dNTPs; 0.1 µg of TS oligonucleotide, 0.1 µg of CX primer; 0.1 mg/ml BSA; 5 µCi of ^{32}P dGTP with a specific activity of 800 Ci/mM; 5 units of Taq polimerase). PCR conditions were 94°C 30 seconds, 50°C 30 seconds, 72°C 90 seconds, 30 cycles. Hot start conditions were used in order to avoid primer dimerization. 5 µl of every reaction were analyzed in 12% acrylamide-bisacrylamide-7 M urea gels, and later detected by autoradiography.

Results showed no telomerase activity in normal mammary gland tissue, and a positive reaction in mammary gland adenocarcinomas (Fig. 2). The intensity of telomerase activity correlated with the protein concentration used in every reaction. Protein concentration has been reported to be critical for telomerase detection and quantitation in mouse tissues[7]. Telomerase activity showed a completely different pattern in the thymic tissue than in mammary gland. Normal thymus had a strong telomerase activity in the three protein dilutions, while thymomas showed a marked reduction in telomerase activity as compared to the normal thymus (Fig. 2). This result was consistently observed, after repeating 3 times telomerase detection in every single sample.

The fact that mammary gland adenocarcinomas have higher telomerase activity than normal mammary tissue is in agreement with the idea that telomerase is involved in the progression of tumor development. However, the detection of lesser telomerase activity in thymomas than in normal thymus was unexpected.

This could be explained by different hypotheses. Thymus is an organ with stem cells and a strong cellular differentiation. Even though thymomas are tumors, they are composed of well differentiated epithelial cells, that could eventually have less telomerase activity than the normal, highly proliferative thymic cells. Another hypothesis is that thymomas produce an inhibitory factor that eventually interferes with telomerase activity. To test this possibility, different concentrations of thymoma extracts were added to normal thymus extracts, then telomerase reaction was carried out. The consequence was a decrease in telomerase activity in the normal thymus extract, observed in 3 independent experiments (Fig. 2). In these 3 experiments, telomerase activity detection was done simultaneously in normal thymus extracts and on thymoma extracts, using the same chemicals and experimental conditions. It has been described that non-especific inhibitors of the PCR step can be present in some mouse tissues[7, 12]. In order to check if these extracts can nonspecifically inhibit the telomerase reaction at the PCR amplification step, thymoma extracts were added to a control PCR reaction that is known to amplify Human Immunodeficiency Virus DNA. No inhibition was observed, and there was no difference between the PCR amplification products with or without thymoma extract (data non shown). These experiments suggest that the lesser telomerase activity

observed in normal thymus extracts after being mixed with thymoma extracts occurs in the extension phase of telomerase reaction.

The results reported here demonstrate that telomerase is present in tumors produced by Polyoma virus in mice. They also show that telomerase activity is both qualitatively and quantitatively different in tumors produced by this DNA-oncogenic virus in 2 different organs in the same animal, and equally suggest that telomerase-inhibitory factors could exist in some tumors.

Recently, It has been demonstrated that transfection of telomerase catalytic subunit into normal (telomerase-negative) human cells produces elongation of telomeres and active cell division, but no cell transformation[13]. This suggests that telomerase expression *per se* is not oncogenic. Moreover, an alternative mechanism of telomere lengthening (ATL) without telomerase activity has been described[14]. These results have been observed in cell cultures, albeit not in tumors. In many reports, telomerase activity has been consistently detected in human[5] and murine[15] tumors, supporting the hypothesis that, somehow, telomerase expression is involved in the development of cancer. Currently, the pathway used by Polyoma to interact with telomerase activity is unknown. It is unclear whether this phenomenon is an early event in tumorigenesis or a late one. The experimental model described here will hopefully allow a better understanding of the conditions whereby telomerase contributes to the tumorigenic process.

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