BIOGERONTOLOGY

Epithalon Inhibits Tumor Growth and Expression of HER-2/neu Oncogene in Breast Tumors in Transgenic Mice Characterized by Accelerated Aging

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Female transgenic FVB mice carrying breast cancer gene HER-2/neu were monthly injected with Vilon or Epithalon (1 μg subcutaneously for 5 consecutive days) starting from the 2nd month of life. Epithalon markedly inhibited neoplasm development: the maximum size of breast adenocarcinomas was 33% lower than in the control ($p<0.05$). The intensity of HER-2/neu mRNA expression in breast tumors of Epithalon-treated mice was 3.7 times lower than in control animals. These results indicate that Epithalon inhibits breast tumor development in transgenic mice, which is probably related to suppression of HER-2/neu expression.

Key Words: *transgenic mice; HER-2/neu; breast cancer; peptides; Epithalon; Vilon*

Interstrain differences in the lifetime and incidence of diseases (*e. g.*, cancer) in mice are genetically determined. There are genes that determine different lifetimes of genetically close strains and genetic stability of animal strains [7,9]. Genetic models of animals with modified lifetimes were elaborated during the past decade. Gerontological studies are now performed on mammals, primarily mice, with spontaneous and induced mutations (knockout and transgenic animals) [3, 9]. Transgenic and knockout mice with short or long lifetimes allow evaluating the role of genes involved in aging in the pathogenesis of age-related diseases, including cancer. The relationship between aging and carcinogenesis attracts much recent attention, which is associated with lengthening of life expectancy and increased proportion of elderly people in many countries [2,3,8].

Transgenic mice carrying the HER-2/neu gene that belongs to the family of tyrosine kinase receptors for epidermal growth factor [1,6] are characterized by high incidence of breast tumors and short lifetime [14]. The average lifetime of virgin FVB/N female mice carrying the HER-2/neu gene is 311± 56 days (maximum lifetime 431 days). Breast adenocarcinomas are found in 80% mice. These animals are characterized by early development of age-related estrous disturbances [3]. The survival of wild-type FVB/N mice aging 24 months is 62%, while the incidence of spontaneous tumors in these animals reaches 66% [12]. Adenomas of the hypophysis and lungs, ovarian tumors, adenomas of the Harderian's glands, lymphomas, histiocytic sarco-

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mas, and pheochromocytomas spontaneously develop in these mice. However, the growth of breast adenocarcinomas in FVB/N mice was not reported.

Recent studies indicate that the pineal indole hormone melatonin inhibits the development of spontaneous and chemical carcinogen-induced breast tumors in laboratory rodents [15]. The peptide preparation from the pineal gland Epithalamin suppresses the growth of spontaneous breast tumors in C3H/Sn and SHR female mice and 7,12-dimethylbenz[a]anthracene-induced breast tumors in female rats [5,11]. The directional synthesis of physiologically active peptides of the pineal gland allowed their introduction into medical practice. Tetrapeptide Epithalon (Ala-Glu-Asp-Gly) was synthesized after amino acid assay of Epithalamin by the method of Khavinson [10]. Lon-term treatment with Epithalon increases the lifetime and inhibits the growth of spontaneous neoplasms in CBA female mice [4]. Here we studied the effects of Epithalon on the development of spontaneous tumors in FVB female mice transfected with the breast cancer gene erbB-2/neu. The synthetic dipeptide Vilon was used for a comparative analysis [13].

MATERIALS AND METHODS

Experiments were performed on homozygote FVB HER-2/neu transgenic FVB/N mice (Charles River) obtained from the Italian National Research Center for Aging and maintained at the Department of Carcinogenesis and Oncogerontology (N. N. Petrov Institute of Oncology). The animals were kept at 22±2°C under a 12-h light/dark regimen and received food and water *ad libitum*. Transgenic females (n=80) were randomly divided into 3 groups. Control animals received monthly subcutaneous injections of 0.1 ml 0.9% NaCl for 5 consecutive days starting from the 2nd month of life. Group 1 and 2 mice were subcutaneously injected with Epithalon and Vilon (1 μg), respectively. These peptides were synthesized at the St. Petersburg Institute of Bioregulation and Gerontology. The development of breast tumors, their localization and size were estimated weakly by palpation. Dead animals were subjected to macro- and microscopic examination after autopsy.

HER-2/neu mRNA expression in transgenic mice was determined by reverse transcription polymerase chain reaction (RT-PCR). Tissue samples were homogenized, and RNA was extracted using Tri-Reagent kits (Sigma Chemical Co.). RNA concentration was measured on a UV1601 spectrophotometer (Scimadzu). cDNA was synthesized by incubation of 0.1 μg RNA with 0.5 mM dNTP, 12.5 ng/μl oligo dT, First Strand buffer containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, and 3 mM $MgCl_2$, reverse transcriptase M-MLV (10 U/μl), RNase inhibitor (1 U/μl), and 0.01 M dithiothreitol (Gibco BRL, final volume 20 μl). Samples were incubated at 37 and 95°C for 1 h and 10 min, respectively. cDNA was frozen at -20°C. PCR was performed by incubating 5 μl cDNA with the reaction mixture containing PCR buffer, 1.5 mM $MgCl_2$, 200 μM dNTP, specific direct and reverse primers (0.8 μM), and Taq DNA polymerase (1 U/μl, Roche Diagnostics GmbH, final volume 50 μl). Samples were incubated in a GeneAmp PCR System 9700 amplifier (Perkin Elmer) to 35 and 30 cycles for HER-2/neu and β-actin, respectively. Primers for HER-2/neu and β-actin were obtained from Roche Diagnostics GmbH. The HER-2/neu fragment of 239 bp had the direct primer 5'-GATCGAATTC CTGGAGGACGTGCCGCTTGTA and reverse primer 5'-GATCAAGCTTATAGCTCCACACATCA CTCTG. The β-actin fragment of 349 bp had the direct primer 5'-TGGAATCCTGTGGCATCCATG

TABLE 1. Effects of Vilon and Epithalon on the Development of Breast Adenocarcinomas and HER-2/neu Oncogene Expression in Transgenic Mice

Parameter	0.9% NaCl (n=28)	Vilon (n=27)	Epithalon (n=25)
Count of mice with breast tumors, %	23 (82)	24 (89)	18 (72)
Development of the 1st tumor, days	168	150	188
Total number of tumors	106	114	85
Count of mice, %			
without tumors	5 (18)	4 (15)	7 (28)**
with 1 tumor	2 (7)	1 (4)	4 (16)**
with 2 and more tumors	21 (75)	22 (81)	14 (56)*
Maximum tumor diameter, cm	2.1±0.2	1.90±0.19	1.40±0.18**
Relative HER-2/neu mRNA expression, arb. U	1.637±0.039	0.829±0.096*	0.445±0.212*

Note. *p<0.001 and **p<0.05 compared to the control.

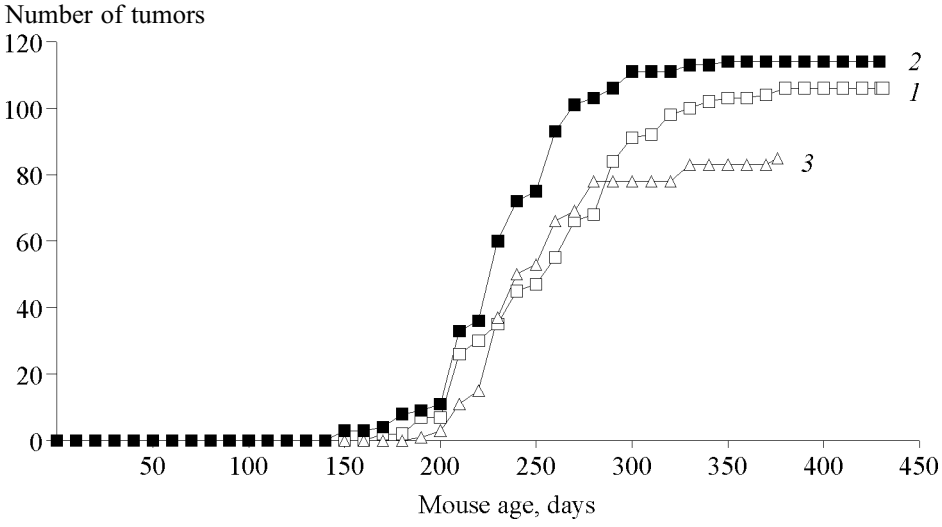

Fig. 1. Effects of Vilon (*2*) and Epithalon (*3*) on the development of breast adenocarcinomas in transgenic HER-2/neu mice (compared to the control, *1*).

AAAC and reverse primer 5'-TAAAACGCAGCT CAGTAACAGTCCG. PCR products and molecular weight standards (DNA molecular weight marker VIII, Roche Diagnostics) were visualized by electrophoresis in 1.5% agarose gel containing 1 μg/μl ethidium bromide. Densitometry was performed on a GelDoc 2000 device (Biorad Laboratories).

The results were analyzed by exact Fischer test, Wilcoxon test, Mann—Whitney test, Student's *t* test, Newman—Keils test, and ANOVA.

RESULTS

In mice receiving Vilon the first breast tumor was detected on day 150 of life. In control animals and mice injected with Epithalon breast tumors developed on days 168 and 188 of life, respectively (Table 1). The dynamics of breast tumor growth did not differ between control and treated mice to the sixth month of life. Then, the incidence of neoplasms markedly decreased in animals injected with Epithalon (Fig. 1). The incidence of breast adenocarcinomas in mice carrying the HER-2/neu gene and receiving Epithalon was lower than in control animals and in mice injected with Vilon (Table 1). In the group treated with Epithalon the number of mice without tumors 1.6-fold surpassed the corresponding parameter in the control group ($p<0.05$). The maximum size of breast adenocarcinomas in mice injected with Epithalon was 33% lower than in the control ($p<0.05$, Table 1).

In mice injected with Epithalon the intensity of HER-2/neu mRNA expression in breast tumors was 3.7 times lower than in control animals (Fig. 2). Vilon decreased the intensity of oncogene expression by 1.97 times compared to the control.

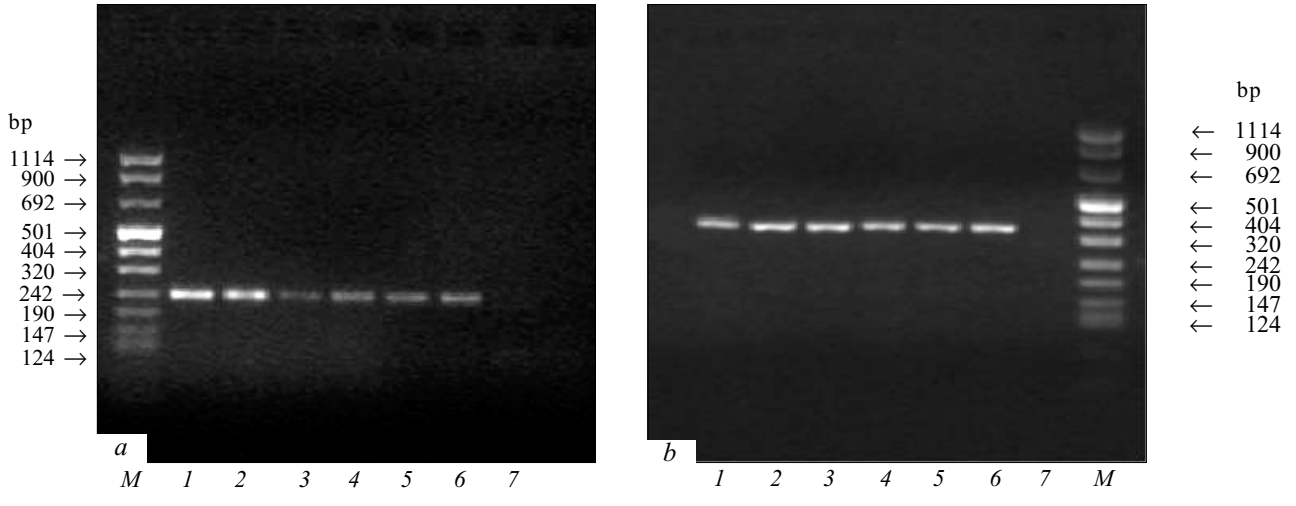

Fig. 2. Expression of HER-2/neu mRNA (*a*) and β-actin (*b*) in breast tumors in FVB/N mice receiving Vilon or Epithalon: 0.9% NaCl (*1*, *2*), Epithalon (*3*, *4*), Vilon (*5*, *6*), and control (*7*).

Our results indicate that Epithalon inhibits breast carcinogenesis and HER-2/neu mRNA expression in transgenic HER-2/neu mice, which is consistent with published data that this tetrapeptide suppresses the development of spontaneous tumors in long-living CBA mice [4]. Moreover, previous studies showed that Epithalamin inhibits the development and growth of spontaneous, carcinogen-induced, and transplanted tumors [5,11].

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