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Transforming Growth Factor-α mRNA and Epidermal Growth Factor Receptor mRNA Expression in Normal and Neoplastic Mammary Glands of Four Strains of Mice with Different Mammary Tumor Potentials

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ABSTRACT—Transforming growth factor-α (TGFα) and epidermal growth factor receptor (EGF-R) mRNAs were determined by reverse transcriptase-polymerase chain reaction (RT-PCR) in the normal and neoplastic mammary glands of four strains of mice with different mammary tumor potentials (from highest to lowest potential): SHN, GR/A, SLN and C3H/He. At 2 months of age, when the mammary glands of these strains consisted mostly of normal tissue, the samples examined showed the positive expressions of both TGFα and EGF-R mRNAs in all strains (4-6 mice per group), except for EGF-R mRNA in the SLN mice, expressed in only 2 of 4 samples associated with no end-bud formation in the mammary glands. At 10 months, all of the samples from all four strains had a positive expression of TGFα mRNA. The EGF-R mRNA expression paralleled the degree of the formation of preneoplastic hyperplastic alveolar nodules (HAN) in all strains. These findings indicate that TGFα and EGF-R participate in the growth of the mammary glands, and that EGF-R especially contributes to the formation of end-buds at younger ages and to that of preneoplastic HAN at later ages. All of the samples of mammary tumors from four strains had positive expressions of both TGFα and EGF-R mRNAs.

INTRODUCTION

Transforming growth factor-α (TGFα) is a 50 amino acid polypeptide (Derynck *et al.*, 1984; Lee *et al.*, 1985) that shares approximately 30% homology with epidermal growth factor (EGF) and acts through binding to EGF receptor (EGF-R) (Todaro *et al.*, 1980; Derynck, 1988). TGFα has been identified in normal mammary glands and mammary tumors *in vivo* and *in vitro* (Pimentel, 1994a; Dickson and Lippman, 1995; Ethier, 1995). Furthermore, Matsui *et al.* (1990), Halter *et al.* (1992) and Mizuno *et al.* (1994) reported the stimulated growth of normal, preneoplastic and neoplastic mammary glands in human TGFα transgenic mice.

EGF-R is a 1,163 amino acid polypeptide, localized on the surface of cells including mammary epithelial cells and mammary tumor cells. EGF-R has tyrosine kinase activity, and closely resembles the oncogene v-erb-B (Pimentel, 1994b). The overexpression of EGF-R in human breast cancer is related to the loss of hormone dependency and poor prognosis (Pimentel, 1994b; Dickson and Lippman, 1995).

Taken together, the above findings indicate the significant participation of TGFα and EGF-R in the normal and neoplastic growth of mammary glands (Kenney and Dickson, 1996).

Four strains of mice maintained in our laboratory are characteristic in having different potentials for normal and neoplastic mammary gland growth higher in order of SHN, GR/A, SLN and C3H/He (Nagasawa *et al.*, 1987a, b).

In this study, the TGFα mRNA and EGF-R mRNA expressions in normal mammary glands and mammary tumors were compared among these four strains of mice.

MATERIALS AND METHODS

Animals

The SHN/Mei, SLN/Mei (Nagasawa *et al.*, 1976; Staats, 1985) GR/AMei (Mühlbock, 1965; Yanai and Nagasawa, 1978) and C3H/HeMei (Nagasawa *et al.*, 1979) strains of mice were used. They were maintained by strict brother × sister mating, kept in aluminum cages (18 × 30 × 15 cm) with wood shavings (M size: CLEA JAPAN, Tokyo, Japan), 4-6 each, maintained in a windowless animal room, which was air-conditioned (21-22°C and 65-70% relative humidity) and artificially illuminated (14 hr of light from 05:00 to 19:00) and ventilated (16 times/hr). The mice were provided with a commercial diet (Lab MR Breeder: Nihon Nosan Kogyo KK, Yokohama, Japan) and tap water *ad libitum*.

In each strain, virgin mice were killed at 2 and 10 months of age by decapitation under light ether anesthesia. The retired mice checked for palpable mammary tumors were also killed in a week after the first tumor appearance (tumor sizes were 3-5 mm).

All procedures were carried out according to the NIH Guide for the Care and Use of Laboratory Animals, USA.

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Normal and preneoplastic mammary gland growth

The unilateral third thoracic gland was fixed in Bouin's solution, prepared for wholemount evaluation and examined under 10-fold magnification. The degree of the formation of normal end-buds was rated from 1 to 7 in increments of 1 (Nagasawa *et al.*, 1980) and the area bound the tops of ducts by the straight lines was also measured automatically by a computerized digitizer (Model LA-525; PLAS, Tokyo, Japan) as an index of duct extension. The number of preneoplastic mammary hyperplastic alveolar nodules (HAN) was counted, and the area of each HAN was also measured by the digitizer.

RNA isolation

Bilateral inguinal mammary glands and a portion of each mammary tumor with the least necrosis were frozen immediately after killing and kept at –70°C. The total RNA in each sample was extracted by the acid guanidinium thiocyanate-phenol-chloroform extraction method (Chomczynski and Sacchi, 1987)

Detection of TGFα mRNA and EGF-R mRNA expressions

The TGFα mRNA and EGF-R mRNA expressions were detected by the reverse transcriptase-polymerase chain reaction (RT-PCR) method. Reverse-transcribed cDNA was synthesized with the use of a cDNA synthesis kit (TaKaRa, Kyoto, Japan) with RAV-2 reverse transcriptase and random primers, and the target cDNA was amplified by PCR with a PCR Amplification kit (TaKaRa) with *Taq* DNA polymerase.

The sense and antisense primers derived from the mouse TGFα cDNA sequence (Vaughan *et al.*, 1992) were used for a PCR reaction for the detection of TGFα mRNA expression. The conditions were the same as detailed previously (Harigaya *et al.*, 1994a).

The PCR reaction for the detection of EGF-R mRNA expression was performed for 40 cycles (1 cycle = 94°C for 1 min, 55°C for 1 min, 72°C for 2 min). The sense and antisense primers derived from the mouse EGF-R cDNA sequence (Avivi *et al.*, 1991; Luetteke *et al.*, 1994) were at nucleotides 236-255 (5'-GGAGGAAAAGAAAGTCT-GCC-3') and 539-520 (5'-CCCATAGTTGGATAGATGG-3'), respectively (Paria *et al.*, 1993). Other conditions were the same as those for the detection of TGFα mRNA expression.

β-Actin primers derived from the mouse sequence (Alonso *et al.*, 1986) were used for the RT-PCR control reaction as reported by Harigaya *et al.* (1994a).

Statistics

The significance of differences in the frequency of TGFα mRNA and EGF-R mRNA expression among the strains was evaluated by χ^2-test. For other parameters, Duncan's Multiple range test was used.

RESULTS

Normal and preneoplastic mammary gland growth (Table 1)

At 2 months of age, mammary rating of SLN was 1-2 indicating that the glands consisted mostly of ducts with no end-buds and it was significantly lower than those of the other three strains (2-4). A significant difference was not seen among the strains in the mammary gland area. The formation of HAN was not detected in any strain.

At 10 months of age, the rating was significantly lower in the SLN and GR/A mice than in the SHN mice. Mammary gland area differed little among the strains.

The number of HAN was the highest in the SHN and the lowest in the SLN and intermediate in the GR/A and C3H/He mice. The number of HAN of the glands with no expression of EGF-R mRNA was low compared to that of the glands with EGF-R mRNA expression; 1.3 ± 0.3 vs. 8, 2 vs. 6.0 ± 0.7 and 2 vs. 12 ± 1.2 in SLN, GR/A and C3H/He, respectively. The mean area of the HAN was significantly lower in the SLN than in the other strains.

TGFα mRNA and EGF-R mRNA expression

The results in normal glands are shown in Table 2 and Fig. 1. At 2 months of age, all samples from each strain expressed TGFα mRNA, and EGF-R mRNA expression was found in all samples from the SHN, GR/A and C3H/He mice, and in 2 of the 4 samples from SLN mice.

At 10 months, all samples from the SHN, SLN and C3H/He mice and 4 of the 5 samples from GR/A mice expressed

Table 1. Normal and preneoplastic mammary gland growth in four strains of virgin mice (Mean ± SEM)

	Number of mice	Normal gland		HAN	
		Rating	Area (mm^2)	Number	Area (mm^2)
2 months					
SHN	5	3.0 ± 0.3^{a}	175 ± 13		
SLN	4	1.8 ± 0.5^{b}	195 ± 11		
GR/A	5	3.1 ± 0.2^{a}	173 ± 17		
C3H/He	5	3.1 ± 0.5^{a}	194 ± 18		
10 months					
SHN	6	2.8 ± 0.3^{a}	227 ± 17	18.2 ± 2.9^{a}	1.05 ± 0.13^{a} (109)*
SLN	5	1.8 ± 0.3^{b}	247 ± 8	3.8 ± 1.6^{c}	0.54 ± 0.06^{b} (19)
GR/A	5	1.8 ± 0.3^{b}	220 ± 21	5.2 ± 0.9^{c}	1.00 ± 0.13^{a} (26)
C3H/He	5	$2.4 \pm 0.3^{a,b}$	249 ± 34	10.0 ± 2.1^{b}	0.90 ± 0.08^{a} (50)

[a, b, c] Values with different superscripts are significantly different at $P<0.05$.

* Number of HAN measured.

Table 2. Frequency of mammary gland expressions of TGFα and EGF-R mRNA in four strains of virgin mice

	2 months		10 months	
	TGFα	EGF-R	TGFα	EGF-R
SHN	6/6[1]	6/6	6/6	6/6[a]
SLN	4/4	2/4	5/5	2/5[b]
GR/A	5/5	5/5	4/5	4/5[a, b]
C3H/He	5/5	5/5	5/5	4/5[a, b]

[1] Number of positive samples/Total number of samples examined.
[a, b] Values with different superscripts are significantly different at $P<0.05$.

Table 3. Frequency of mammary tumor expression of TGFα mRNA and EGF-R mRNA in four strains of retird mice

	SHN	SLN	GR/A	C3H/He
TGFα	5/5[1]	3/3	3/3	5/5
EGF-R	5/5	3/3	3/3	5/5

[1] Number of positive samples/Total number of samples examined.

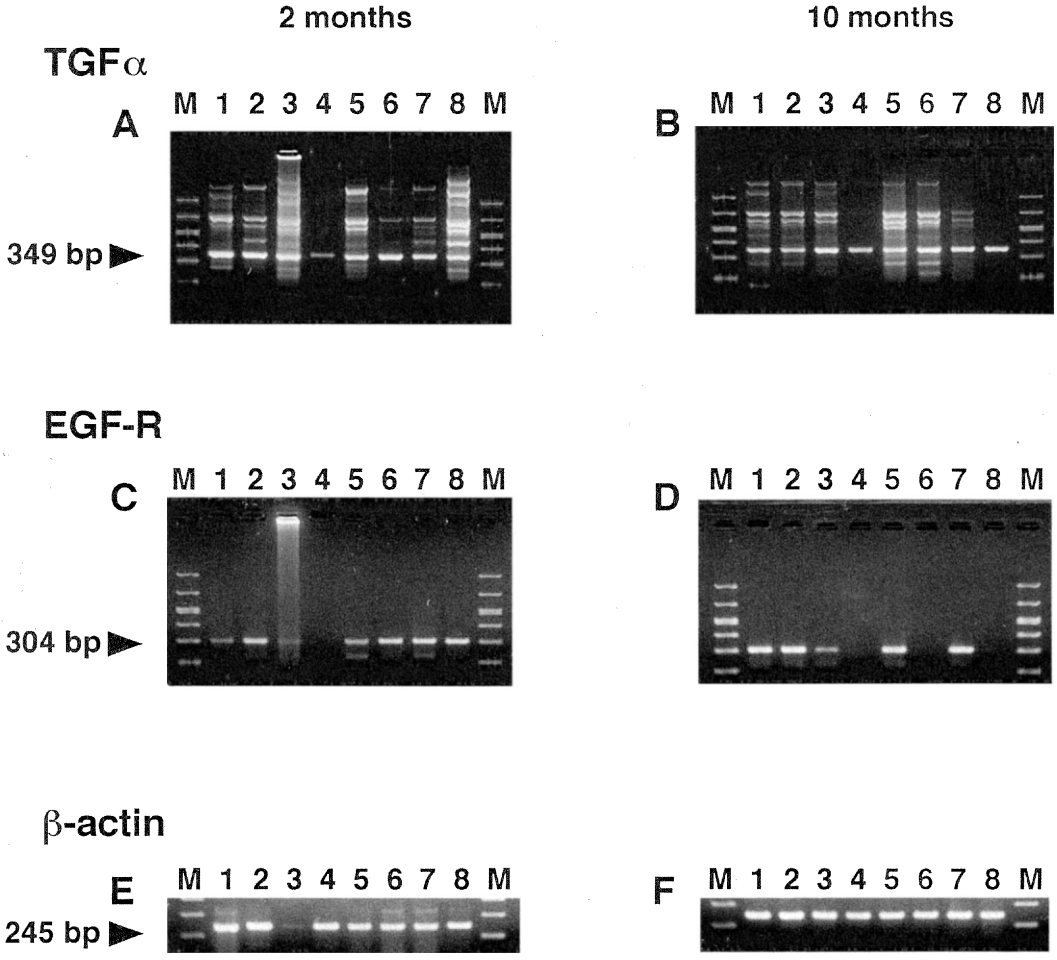

Fig. 1. Agarose gel electrophoresis of RT-PCR products in four strains of virgin mice. The PCR products of the target sequences for TGFα, EGF-R and β-actin were 349 bp, 304 bp and 245 bp, respectively. Lane M, Molecular size of marker; Lanes 1 and 2, SHN; Lanes 3 and 4, SLN; Lanes 5 and 6, GR/A; Lanes 7 and 8, C3H/He.

TGFα mRNA. EGF-R mRNA expression was found in all samples from the SHN mice and in 2 of the 5 samples from SLN and 4 of the 5 from the GR/A and C3H/He mice. The difference in EGF-R mRNA expression between the SHN and SLN mice was significant.

As shown in Table 3, all mammary tumor samples from each strain expressed both TGFα mRNA and EGF-R mRNA at 10 months of age.

DISCUSSION

At 2 months of age, when the mammary glands of the mice consisted mostly of normal tissue without HAN, TGFα mRNA expression was seen in all samples from all four strains of virgin mice. BALB/c and ICR virgin mice, which have no mammary tumor potentials, were also shown to express TGFα mRNA in the mammary glands at about 1.8-3 months of age

(Snedeker *et al.*, 1991; Yasuda *et al.*, 1996). These results suggest that TGFα mRNA expression does not directly relate to whether or not the mouse strain has a mammary tumor potential, and that TGFα participates mostly in normal gland growth at these ages.

EGF-R mRNA expression was also seen in all of the samples from SHN, GR/A and C3H/He mice, but in only 2 of the 4 samples from SLN mice. The mammary glands of the SLN mice consisted mostly of ducts with no end buds, while those of the other 3 strains contained several end-buds. Snedeker *et al.* (1991) reported the participation of EGF-R in the end-bud formation in ovariectomized virgin BALB/c mice at about 3 months of age. Taken together, these findings suggest that the EGF-R at the developmental stage of mammary gland acts specifically on mammary end-bud formation with little relationship to whether the mouse has a mammary tumor potential.

At 10 months, TGFα mRNA expression was seen in almost all mammary gland samples from the four strains of virgin mice examined. Only 1 of the 7 samples from ICR virgin mice expressed TGFα mRNA at about 7 months of age (Yasuda *et al.*, 1996). Thus, the mammary gland expression of TGFα mRNA in aging mice is throuht to be higher in strains with a mammary tumor potential than in those without it.

EGF-R mRNA expression was also observed in all of the samples from the SHN mice, but in only 2, 4 and 4 out of 5 samples from the SLN, GR/A and C3H/He mice, respectively. The mammary glands in all strains at 10 months contained preneoplastic HAN. However, in the 3 samples from SLN mice and 1 each from GR/A and C3H/He mice, in which mammary EGF-R mRNA expression was not detected, HAN formation was low compared to the mammary glands with positive expression. This result suggests that EGF-R mRNA is one of the enhancing factors for the formation of HAN and/or its transformation into the tumor.

All of the mammary tumor samples from the four strains showed positive TGFα mRNA expression in this study. This finding is in good agreement with our previous study (Harigaya *et al.*, 1994b). Furthermore, EGF-R mRNA was also detected in all samples examined. This may reflect the hormone independency or autonomy even in the early stage of progression and the marked malignancy of these mouse mammary tumors. It has been reported that in human mammary tumors, EGF-R expression is inversely related to hormone dependency and is thus a sign of poor prognosis (Pimentel, 1994a; Dickson and Lippman, 1995).

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