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Transformation of mouse mammary epithelial cells with the Ha-*ras* but not with the *neu* oncogene results in a gene dosage-dependent increase in transforming growth factor-α production

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An enhanced expression of transforming growth factor-α (TGFα) was demonstrated in two clones of NOG-8 mouse mammary epithelial cells, NOG-8 SR1 and NOG-8 SR2, that have been transformed by a v-Ha-*ras* oncogene. The amount of TGFα production in NOG-8 SR1 and NOG-8 SR2 cells was dependent on the level of $p21^{ras}$ expression in these clones, which directly correlated with their cloning efficiency in soft agar. There was also a decrease in the number of epidermal growth factor (EGF) receptors on the NOG-8 SR1 and NOG-8 SR2 cells that is proportional to the amount of TGFα secreted. These effects were specific for *ras* because *neu*-transformed NOG-8 cells grew in soft agar at a comparable level to NOG-8 SR2 cells yet did not show any increase in TGFα production or change in EGF receptor expression.

Oncogene; Transformation; Oncogene, *ras*; Oncogene, *neu*; Transforming growth factor-α

1. INTRODUCTION

Transforming growth factor-α (TGFα) is a growth factor that is a mitogenic peptide and that is structurally and functionally related to epidermal growth factor (EGF) [1]. In this respect, TGFα binds to the EGF receptor (c-*erbB*) and activates the EGF receptor tyrosine kinase [1,2]. Elevated levels of TGFα have been found in a number of rodent and human breast carcinomas [3–6]. TGFα expression can be enhanced by estrogens in estrogen-responsive rat and human breast tumor cell lines [4–6]. In addition, we have demonstrated previously that transformation of two different mouse mammary epithelial cell lines with an activated c-Ha-*ras* proto-oncogene induces an increase in both TGFα mRNA expression and TGFα protein production [7,8]. In fact, the increase in TGFα synthesis in *ras*-transformed NOG-8 mouse mammary epithelial cells is coordinately and temporally coupled to a change in *ras* gene expression [8]. These results collectively suggest that TGFα may be functioning as an autocrine growth factor for breast cancer cells and that TGFα may be acting as an intermediary in the transformation pathway for an activated *ras* proto-oncogene in mammary epithelial cells [9,10].

The present study was undertaken to determine if there is a gene-dosage effect of $p21^{ras}$ expression on TGFα production in two different clones of v-Ha-*ras*-transformed NOG-8 mouse mammary epithelial cells and to ascertain if this effect was specific for *ras*-transformed mouse mammary cells or if it can be detected in NOG-8 cells that have been transformed by an entirely different activated proto-oncogene such as *neu* (*erbB*-2). The *neu* gene

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Abbreviations: TGFα, transforming growth factor-α; EGF, epidermal growth factor; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; CM, conditioned medium; RIA, radioimmunoassay; RRA, radioreceptor assay

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was selected because the p185^{neu} protein is homologous to the EGF receptor and because there is a substantial body of clinical evidence demonstrating that amplification and/or overexpression of this proto-oncogene is associated with a subset of more aggressive primary human breast tumors [11–13].

2. MATERIALS AND METHODS

NOG-8 cells are an epithelial subclone of a nontumorigenic mouse mammary cell line, NMuMg, originally isolated from the mammary gland of NaMru mice [14]. NOG-8 SR1 and NOG-8 SR2 cells are two different clones of NOG-8 cells that were derived after infection with the recombinant retroviral pZSR vector containing the murine Harvey sarcoma virus v-Ha-*ras* oncogene and the neomycin resistance gene and after selection for G418 resistance, as previously described [15]. NOG-8 *neu* cells are a G418-resistant population of NOG-8 cells that had been cotransfected after calcium phosphate precipitation with an expression vector plasmid containing the point-mutated rat *neu* proto-oncogene linked to the SV40 early promoter and the pSV2neo plasmid [14]. The cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin (GIBCO, Grand Island, NY) in a 5% CO_2 humidified incubator at 37°C.

For soft agar growth, 2×10^4 cells/35-mm dish were seeded into 0.3% Difco Noble agar (Difco, Detroit, MI) supplemented with DMEM containing 10% FBS, layered over a base layer of 0.8% agar medium. After 12 days, cells were stained with nitro blue tetrazolium (Sigma Co., St. Louis, MO), and colonies larger than 50 μm were counted with an Artek 880 colony counter (Artek System, Farmingdale, NY).

Preparation of conditioned medium (CM) that was collected over a 48-h period from cultured cells and determination of TGFα protein by radioimmunoassay (RIA) and by radioreceptor assay (RRA) were performed as previously described [5,8].

Western blot analysis of the p21^{ras} protein was performed using the rat monoclonal anti-p21^{ras} antibody, Y13-259, as previously described [16].

Western blot analysis of the p185^{neu} protein was carried out using 21N, a rabbit polyclonal anti-*neu* anti-serum that was raised against a synthetic peptide corresponding to the C-terminal region of the human c-*erbB*-2 protein (residues 1243–1255) as previously described [17].

The specific binding of ^{125}I-EGF was performed on cells in monolayer culture using mouse ^{125}I-EGF (spec. act. 100 μCi/μg, Amersham, Arlington Heights, IL) as previously described [7]. The number of EGF-binding sites and the K_ds for binding were determined by Scatchard analysis of the specific-binding isotherms [8].

Poly(A)$^+$ RNA was isolated from the cells, and equivalent amounts (10 μg/lane) were electrophoresed through a denaturating 1.2% agarose/2.2 M formaldehyde gel [5]. Ethidium bromide staining of the gels before blotting demonstrated that each lane contained equivalent amounts of RNA. The RNA was then transferred by capillary blotting to a Biotrans nylon membrane (ICN Biomedicals, Costa Mesa, CA) [18] and hybridized sequentially as previously described [8] to the following ^{32}P-labeled, nick-translated cDNA probes: a 6.6-kb *Bam*HI fragment of a human c-Ha-*ras* cDNA probe [19]; a 406-bp *Eco*RI-*Apa*I restriction fragment derived from a human TGFα cDNA clone, pTGF-C1 [20]; a 6.0-kb *Eco*RI/*Hin*dIII fragment of the human c-*myc* cDNA probe [21]; and a 770-bp human β-actin cDNA probe (Oncor, Gaithersburg, MD).

3. RESULTS AND DISCUSSION

Infection of primary mouse mammary epithelial cells or established NOG-8 mouse mammary epithelial cells with the recombinant pZSR virus containing the viral Ha-*ras* oncogene results in the anchorage-independent growth of these cells in soft agar and their ability to form undifferentiated carcinomas in nude mice [15]. Several independent clones of v-Ha-*ras*-infected NOG-8 cells were isolated and expanded after G418 selection. Although uninfected NOG-8 cells failed to grow in soft agar, two of these v-Ha-*ras*-infected clones, NOG-8 SR1 and NOG-8 SR2, exhibited soft agar colony forming efficiencies ranging from approximately 7.5% for NOG-8 SR1 cells to 15% for NOG-8 SR2 cells (fig.1A). NOG-8 cells transfected with an activated rat c-*neu* gene also grew aggressively in soft agar and exhibited a cloning efficiency that was comparable to the v-Ha-*ras*-

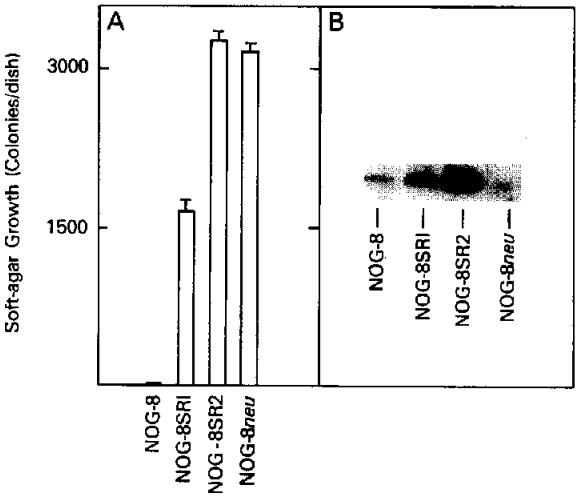

Fig.1. (A) Anchorage-independent growth in soft agar of v-Ha-*ras*- and *neu*-transformed NOG-8 cell lines. Data represent an average (± SD) of four experiments in quadruplicate. (B) Western blotting of p21^{ras} levels in v-Ha-*ras*- and *neu*-transformed NOG-8 cell lines.

transformed NOG-8 SR2 cells. After injection of 1×10^6 cells in the dorsal flank of 4- to 6-week-old nude mice, NOG-8 SR1 cells formed tumors in 100% of the animals within 6–7 weeks whereas NOG-8 SR2 cells formed tumors in all of the mice after only 2 weeks [15]. The *neu*-transfected NOG-8 cells formed histologically comparable tumors as v-Ha-*ras*-transformed NOG-8 cells. Injection of a comparable number of NOG-8 *neu* cells led to the development of tumors in two of two nude mice within 3–4 weeks.

The relative levels of total endogenous c-Ha-*ras* and v-Ha-*ras* protein in these cells was determined by Western blot analysis and subsequent densitometric scanning. As shown in fig.1B, NOG-8 SR1 cells expressed approximately 6–8-fold more $p21^{ras}$ protein than the uninfected, parental NOG-8 cells, which expressed low levels of endogenous $p21^{ras}$. NOG-8 SR2 cells exhibited approximately 30-fold more $p21^{ras}$ than the NOG-8 cells, whereas little or no $p21^{ras}$ was detected in the NOG-8 *neu* cells. NOG-8 *neu* cells produced elevated amounts of $p185^{neu}$ as determined by Western analysis (data not shown). In contrast, no $p185^{neu}$ was found in the NOG-8 cells. Northern blot analysis demonstrated the presence of a 4.8-kb pZSR viral transcript in the NOG-8 SR1 and NOG-8 SR2 cells but not in the uninfected NOG-8 or NOG-8 *neu* cells (fig.2A). In fact, the relative level of expression of this transcript correlated reasonably well with the amount of $p21^{ras}$ protein that was present in these two v-Ha-*ras*-transformed NOG-8 clones.

Enhanced production and secretion of TGFα are frequently associated with rodent fibroblasts and epithelial cells that have been transformed with an activated c-Ha-*ras* proto-oncogene and v-Ki-*ras* or v-Ha-*ras* oncogenes [7,8,22,23]. To ascertain if there was any relationship among the level of v-Ha-*ras* expression, soft agar growth and TGFα production, CM from these cell lines was analyzed for immunoreactive and biologically active TGFα in a RIA and RRA, respectively. As shown in fig.3, the levels of biologically active and immunoreactive TGFα were equivalent within each of the cell lines. NOG-8 cells secreted low levels of TGFα (5–10 ng/10^8 cells/48 h), whereas NOG-8 SR1 cells produced 4–5-fold (35–40 ng/10^8 cells/48 h) and NOG-8 SR2 cells produced 8–10-fold (80–90 ng/10^8 cells/48 h) higher levels of TGFα than NOG-8 cells. In contrast, NOG-8 *neu* cells did not show any change in TGFα production compared with NOG-8 cells. These differences in secreted TGFα are reflected by comparable

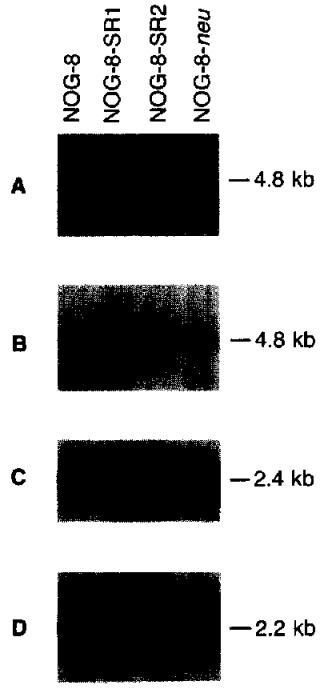

Fig.2. Northern blot analysis of v-Ha-*ras* mRNA (A), TGFα mRNA (B), β-actin mRNA (C) and c-*myc* mRNA (D) in v-Ha-*ras*- and *neu*-transformed NOG-8 cell lines.

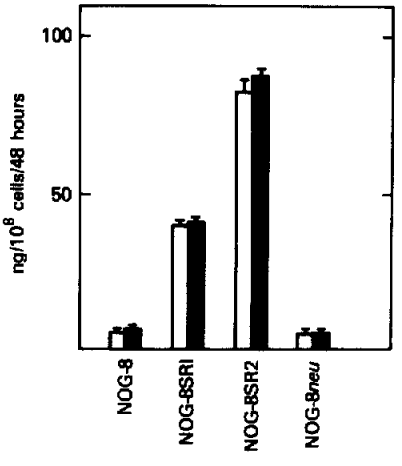

Fig.3. Immunoreactive (□) and biologically active (■) TGFα protein levels in the CM obtained from v-Ha-*ras*- and *neu*-transformed cell lines. Data represent an average (±SD) of three experiments in duplicate.

changes in the amount of a 4.8-kb TGFα-specific mRNA transcript that can be detected in these cell lines (fig.2B). In fact, the differences in TGFα mRNA expression that are observed in the individual NOG-8 cell lines are not due to variations in the amount of RNA that is loaded on the gels because all the cells expressed a comparable level of β-actin mRNA (fib.2C). In addition, the change in TGFα mRNA expression that is observed after *ras* transformation is specific for this growth factor because the levels of c-*myc* mRNA were approximately equivalent (fig.2D).

One consequence of an increase in TGFα production and secretion in a number of different *ras*-transformed cells is a reduction in the number of EGF receptors that can be measured on the surface of these cells [7,8,24]. This phenomenon is probably due in large part to a chronic occupation of these receptors by endogenously produced TGFα, which results in a down-regulation of these receptors [1,7,8,22]. Fig.4 demonstrates the specific binding of different concentrations of ^{125}I-EGF to the v-Ha-*ras*-transformed NOG-8 SR1 and SR2 clones and to NOG-8 *neu*-transformed cells. Saturation of specific binding in all the cell lines occurred between 10 and 25 ng/ml of ^{125}I-EGF. NOG-8 cells and transformed NOG-8 *neu* cells bound approximately equal amounts of ^{125}I-EGF and possessed between 90000 and 95000 EGF receptor sites/cell as determined by Scatchard analysis (fig.4, inset). In contrast, NOG-8 SR2 cells showed a 90% reduction in the number of EGF receptor sites (~12000 sites/cell) while NOG-8 SR1 cells exhibited almost a 50% reduction in EGF receptor sites (47000 sites/cell) compared with NOG-8 cells. All of the cell lines except the NOG-8 SR2 cells showed at least two classes of EGF receptor sites with a high affinity of 2.5×10^{-10} M and a low affinity of 1×10^{-9} M. NOG-8 SR2 cells lacked the high-affinity class of receptor sites, which is in agreement with previous studies showing that high levels of *ras* expression or TGFα production have to be reached before there is a loss in the high-affinity population of EGF receptors [8,25].

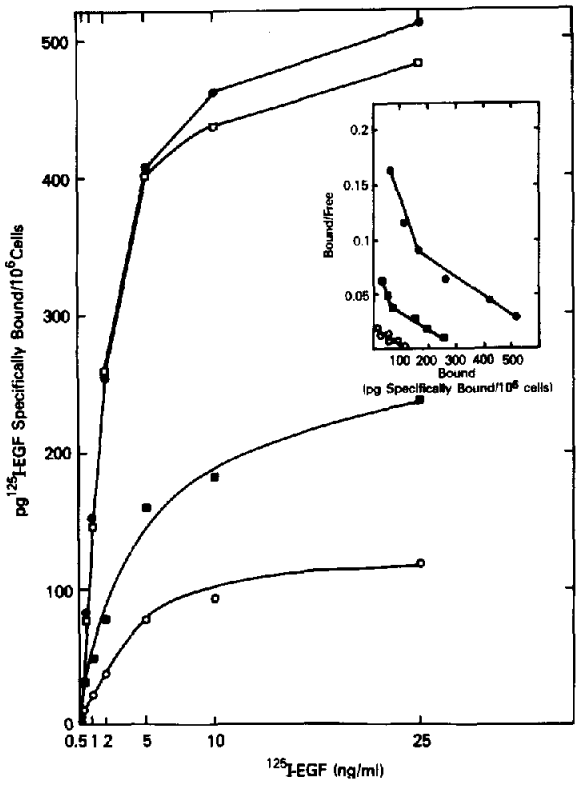

Fig.4. ^{125}I-EGF specific binding to NOG-8 cells (●—●), NOG-8 SR1 cells (■—■), NOG-8 SR2 cells (○—○) and NOG-8 *neu* cells (□—□). (Inset) Scatchard plots of the binding isotherms. Data represent an average of three experiments in duplicate. The SD was less than 10%.

The results of this study demonstrate that the amount of TGFα produced in Ha-*ras*-transformed mammary epithelial cells is dependent on the levels of p21^{ras} expression in these cells. This may have some clinical significance because an enhanced expression of p21$^{c\text{-}Ha\text{-}ras}$ and c-Ha-*ras* mRNA has been detected in a majority of primary human breast tumors [26,27]. Furthermore, the levels of secreted TGFα can be correlated reasonably well with the cloning efficiency of these cells in soft agar, which is an accurate in vitro index of their tumorigenicity. Specifically, NOG-8 SR2 cells expressed approximately 3-4-fold more p21^{ras} than NOG-8 SR1 cells and exhibited twice the soft agar colony forming ability as NOG-8 SR1 cells. These differences are reflected by a 2-fold higher level of TGFα production in the NOG-8 SR2 cells compared with the NOG-8 SR1 cells. These results support a role for TGFα in the transformation of mammary epithelial cells that can be produced by an activated *ras* gene and further suggest that TGFα production is strongly coupled to a regulation of *ras* gene expression. In addition, this effect

is apparently specific for *ras* because *neu*-transformed NOG-8 mammary epithelial cells failed to show any change in TGFα production, although they are equally as efficient as *ras*-transformed NOG-8 cells for cloning in soft agar and for forming tumors in nude mice. This suggests that *neu* may function through a different transformation pathway than *ras*, at least with respect to the potential involvement of TGFα.

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