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HORMONE-REGULATED EXPRESSION OF CELLULAR *ras*^{H} ONCOGENE IN MAMMARY CARCINOMAS IN RATS

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SUMMARY: Differential gene expression has been observed in hormone-dependent rat mammary carcinomas during their growth and regression. A 22K MW protein, a prominent *in vitro* translation product of the growing as compared to the regressing tumor, was identified as the c-*ras*^{H}-21,000-dalton transforming protein (p21) using a monoclonal antibody that reacts specifically with Harvey-related p21 species. The amount of p21-translated protein sharply decreased in the translation products of the regressing tumors within 6 hours post ovariectomy or dibutyryl cyclic AMP treatment. The results show that an enhanced expression of the c-*ras*^{H} oncogene is associated with hormone-dependent growth of mammary carcinomas *in vivo* and that suppression of this oncogene precedes the tumor regression induced by either hormone withdrawal or dibutyryl cyclic AMP treatment.

INTRODUCTION: A single feeding of 7,12-dimethylbenz(α)anthracene (DMBA) induces primary mammary carcinomas in female Sprague-Dawley rats (1). The mammary carcinomas produced by this procedure possess physiological characteristics of hormone dependence; the tumors shrink in size after ovariectomy just as the normal mammary glands of the host undergo atrophy after removal of the ovaries (1). The injections of $N^{6},O^{2'}$-dibutyryl cyclic adenosine 3',5'-monophosphate (dibutyryl cyclic AMP) in the host also induce regression of the tumors, as does ovariectomy (2).

We have shown previously (3, 4), that cyclic AMP-binding activity is inversely related to estrogen-binding activity in hormone-dependent mammary tumors during their growth and regression. Moreover, translocation of cytoplasmic cyclic AMP-binding protein and protein kinase into the nucleus and new phosphorylation of a nuclear protein occur in the regressing tumors after either hormone-removal (ovariectomy) or dibutyryl cyclic AMP treatment (3-5).

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In a recent report (6) we have further shown that growth and regression of DMBA-induced mammary carcinomas may correlate with the changes in gene expression and that estrogen and cyclic AMP antagonism may be involved in this gene expression. When poly(A)+ RNAs isolated from growing and regressing DMBA tumors were used in an *in vitro* protein-synthesizing system, two prominent translated proteins with MW of 35K and 22K were found in the growing tumors, and the amounts of these translated proteins sharply decreased in the regressing tumors following either ovariectomy or dibutyryl cyclic AMP treatment (6). The results suggested that an enhanced expression and suppression of genes associated with cellular proliferation or oncogenic expression may be involved in the growth/regression of mammary tumors.

In this report we present data showing that expression of the cellular proto-oncogene *ras* (c-*ras*), which may be homologous to the transforming gene of Harvey sarcoma virus (v-*ras*^{H}) (7), is enhanced during growth of a hormone-dependent mammary tumor and that suppression of this oncogene precedes tumor regression.

MATERIALS AND METHODS: Primary, DMBA-induced (1) mammary carcinomas (2-3 g) in Sprague-Dawley female rats (200-250 g) were used. Tumor growth was monitored by daily measurements as described in (2). Regression of tumors was induced by either ovariectomy (under anesthesia) or dibutyryl cyclic AMP treatment (10 mg/day/200 g rat, s.c.) (2). By day 3, ovariectomy and dibutyryl cyclic AMP treatment each produced regression (> 20% decrease in volume) in 86% of the tumors. Total RNA from tumors was extracted as described by Deeley *et al.* (8). Poly(A)-containing RNAs were isolated using a poly(U)-Sepharose affinity column (9). Rabbit reticulocyte lysate (Amersham Corp., Arlington Heights, IL) was used for cell-free protein translation and protein synthesis was carried out as described previously (6). Immunoprecipitation of p21 was carried out by the method of Furth *et al.* (10).

RESULTS AND DISCUSSION: We examined whether the 22K protein, prominent *in vitro* translated proteins of the growing as compared to the regressing tumors (6), represents the 21,000-dalton transforming gene product (p21) of c-*ras* proto-oncogene (7, 11-13). The [^{35}S]methionine-labeled translation products from DMBA tumors were analyzed for p21 by immunoprecipitation with monoclonal antibody #259 (10) directed against the Harvey sarcoma virus-encoded p21. As shown in Fig. 1, a substantial amount of p21 was immunoprecipitated from the translation products of growing tumors (lane 4) and the p21 protein band co-

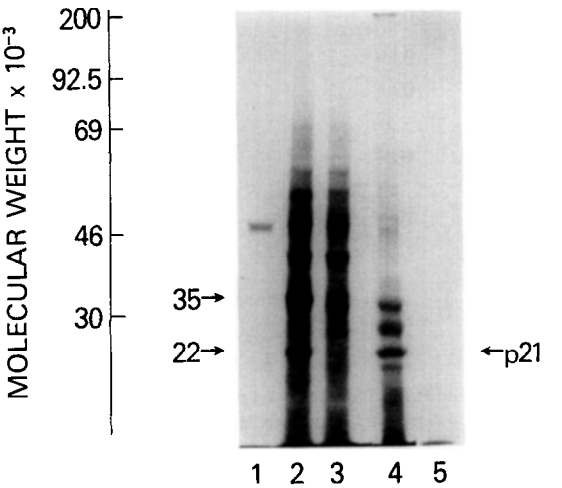

Fig. 1. Autoradiograph showing immunoprecipitation of 21,000-dalton transforming protein (p21) from the in vitro translation products of DMBA-induced mammary tumors. Four to six pooled, growing (> 30% increase in volume) or regressing (> 20% decrease in volume) tumors quickly frozen in liquid nitrogen were used for each poly(A)+ RNA preparation. [^{35}S]methionine-labeled in vitro translation products of growing and regressing tumors were immunoprecipitated with monoclonal antibody #259 (10) directed against the Harvey sarcoma virus-encoded p21. Immunoprecipitates were dissolved and subjected to electrophoresis on 0.1% sodium dodecyl sulfate/10% polyacrylamide gels as described in (16). (1) no messenger; (2) and (3) in vitro translation products from mRNAs of growing and regressing (3 days post ovariectomy) tumors, respectively; (4) and (5) p21 immunoprecipitates of (2) and (3), respectively. The data represent one of five experiments that gave essentially the same results.

migrated with the 22K protein band (compare lanes 2 and 4). Along with the p21 protein, the antibody also immunoprecipitated two other proteins with MW of 30K and 35K (lane 4). The 22K, 30K, and 35K proteins were absent when immunoprecipitations were carried out with the control serum, indicating that these proteins are probably the specific products of the ras gene. The significance of the 30K and 35K protein immunoprecipitation is, however, not clear at present, since it has been indicated (11, 12) that p21 is the only protein definitely encoded by the viral ras gene. The 30K and 35K proteins may represent the tumor ras gene-specific products since using the same methodology we detected p21 but not 30K and 35K proteins in a cultured cell transformed by v-ras^{H} gene (Clair and Cho-Chung, unpublished data). The 22K protein decreases markedly in the translation products of mRNAs from the regressing tumors following either ovariectomy or dibutyryl cyclic AMP treatment [lane 3 (6)]. The antibody detected no significant amount of p21 in the translation products of mRNAs from

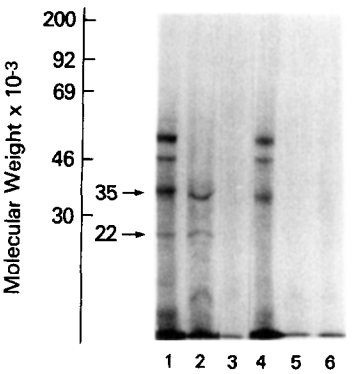

Fig. 2. Autoradiograph showing immunoprecipitation of p21 from in vitro translation products of the growing and regressing DMBA mammary carcinomas using a monoclonal antibody 238 (10) specific for ras^H-p21 species. (1) in vitro translation products from growing tumor; (2) p21 immunoprecipitates of (1); (3) immunoprecipitation of (1) with the control serum; (4) in vitro translation products from regressing tumor 6 hours post dibutyryl cyclic AMP treatment; (5) p21 immunoprecipitates of (4); (6) p21 immunoprecipitates of in vitro translation products of regressing tumor 6 hours post ovariectomy. The data represents one of three experiments that gave essentially the same results.

the regressing tumors (lane 5). These data suggested that expression and suppression of a c-ras oncogene is involved in the growth and regression, respectively, of a hormone-dependent mammary carcinoma.

In the rat, ras is a family of genes consisting of at least two genes coding for p21 protein (14). These genes have been acquired by Harvey (HaMuSV) and Kirsten (KiMuSV) sarcoma viruses by passage of murine leukemia viruses through rat cells (15). We examined whether the p21 expressed in DMBA-induced mammary tumors is related to the products of v-ras^H or v-ras^K. Fig. 2 shows the data on the immunoprecipitation using the monoclonal antibody #238 (10) that reacts with Harvey-related p21 but not with Kirsten-related p21 species. The antibody specifically immunoprecipitated the 22K and 35K proteins from the translation products of the growing tumors (lane 2). The results suggested that the tumor p21 may be the product of the c-ras^H gene. As early as 6 hours post ovariectomy or dibutyryl cyclic AMP treatment when there is no appreciable change in tumor size, the expression of p21 sharply decreased in the tumors (Fig. 2, lanes 5 and 6), indicating that the decrease in p21 expression is an early event of tumor regression.

p21 expression in growing and regressing tumors, and in normal mammary glands of Sprague-Dawley rats, was estimated quantitatively. It can be seen

Table I

mRNA from	Relative level of *in vitro* translated p21
Growing DMBA tumor	10.0 ± 2.0
Regressing DMBA tumor (3 days post ovariectomy)	1.5 ± 0.2
Regressing DMBA tumor (3 days post dibutyryl cyclic AMP treatment)	1.5 ± 0.2
Mammary gland of virgin rat	1.0 ± 0.2

Quantitation of *in vitro* translated p21 from mRNAs of growing and regressing DMBA tumors and mammary glands of Sprague-Dawley virgin rats (100 days old). The levels of p21 protein were quantified from densitometric tracings of autoradiographs of gels after immunoprecipitation of the *in vitro* translation products with anti-p21 monoclonal antibody #259 (10). The levels of p21 protein translated from the growing and regressing tumor mRNAs are expressed relative to the level of p21 translated from mRNA of virgin mammary gland. The data represent average values ± S.E. of six experiments and each mRNA preparation represents four to six pooled tumors or tissues.

in Table I that the levels of p21 in the translation products of the regressing tumors at day 3 after ovariectomy or dibutyryl cyclic AMP treatment were only 15% of those found in the translation products of the growing tumors. Table I also shows that the levels of p21 in the translation products from the growing tumor were tenfold greater than those found in the mammary glands, suggesting that the growing tumors may contain an increased amount of p21 mRNA. Approximately 12% of DMBA-induced tumors that have failed to regress and continued to grow after ovariectomy or dibutyryl cyclic AMP treatment contained the levels of translated p21 as low as that in virgin mammary glands and the p21 levels did not change after either ovariectomy or dibutyryl cyclic AMP treatment (data not shown). Thus, the change in translated p21 level observed during growth and regression appears to be specifically related to the hormone dependency of mammary tumors.

The altered levels of translated proteins between growing and regressing tumors could be due to either altering in the transcription rate or differences in the stabilization or degradation of the mRNAs. Our finding that both dibu-

tyryl cyclic AMP treatment and ovariectomy induced the same decrease in p21-translated protein favors the possibility of the former: it seems unlikely that molecules as different from each other as are estrogen and cyclic AMP could each be involved in the stabilization and degradation, respectively, of the same message.

Quantitative and qualitative activation of cellular oncogenes have been shown in neoplastic cells (17). In the hormone-dependent mammary carcinomas described above, p21 expression is enhanced during growth and is suppressed during regression, suggesting that the changes in the c-*ras* gene expression may be due to quantitative modulation of this gene at a regulatory locus. Importantly, either hormone withdrawal (ovariectomy) or dibutyryl cyclic AMP treatment results in suppression of the *ras* gene expression in the tumors and the tumors eventually regress. It is probable that an antagonistic interaction between estrogen and cyclic AMP, as proposed earlier (18), might be involved in the enhancement and suppression of the *ras* gene expression in growth/regression of mammary tumors.

This study presents the first evidence that hormone-stimulated expression of cellular *ras* oncogene is associated with the growth of mammary carcinoma. The *ras* expression in DMBA-induced tumors was tenfold higher than that in the virgin mammary gland. That hormone withdrawal (ovariectomy) induces decrease in the c-*ras* gene expression in the regressing tumors suggests a possible causal role for this oncogene in the hormone-dependent mammary tumors. Indeed, an amplified expression of the 22K protein, identified as p21 in DMBA tumors, has also been found in another hormone-dependent mammary tumor, MTW9 (19). In hormone-independent mammary tumors, the expression of p21 was not elevated. In a recent report, Sukumar *et al.* (20) have shown that N-nitroso-N-methylurea-induced rat mammary carcinomas, which do not regress and continue to grow upon ovariectomy, contain a structurally altered c-ras^{H} gene that exhibits a high transforming activity. It is conceivable that loss of hormonal regulation and acquisition of genomic alteration of c-ras^{H} might be correlated with hormone-independent growth of mammary tumors.

ras genes are widely conserved in various organisms and are members of a family of related human genes (15). The involvement of this gene in the hormone-stimulated growth of rat mammary carcinomas suggests the possibility that this oncogene might play a role in the growth of hormone-dependent breast cancer in humans.

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REFERENCES

1. Huggins, C. and Yang, N.C. (1962) Science 137, 257.
2. Cho-Chung, Y.S. and Gullino, P.M. (1974) Science 183, 87.
3. Bodwin, J.S., Clair, T. and Cho-Chung, Y.S. (1978) Cancer Res. 38, 3410.
4. Cho-Chung, Y.S. (1980) J. Cyclic Nucleotide Res. 6, 163.
5. Cho-Chung, Y.S. and Redler, B.H. (1977) Science 197, 272.
6. Huang, F.L. and Cho-Chung, Y.S. (1982) Biochem. Biophy. Res. Commun. 107, 411.
7. Ellis, R.W., DeFeo, D., Maryak, J.M., Young, H.A., Shih, T.Y., Chang, E.H., Lowy, D.R. and Scolnick, E.M. (1980) J. Virol. 36, 408.
8. Deeley, R.G., Gordon, J.I., Burns, A.T.H., Mullinix, K.P., Bina-Stein, M. and Goldberger, R.F. (1977) J. Biol. Chem. 252, 8310.
9. Firtel, R.A. and Lodish, H.F. (1973) J. Mol. Biol. 79, 295.
10. Furth, M.E., Davis, L.J., Fleurdelys, B. and Scolnick, E.M. (1982) J. Virol. 43, 294.
11. Shih, T.Y., Weeks, M.O., Young, H.A. and Scolnick, E.M. (1979) Virology 96, 64.
12. Shih, T.Y., Weeks, M.O., Young, H.A. and Scolnick, E.M. (1979) J. Virol. 31, 546.
13. Langebeheim, H., Shih, T.Y. and Scolnick, E.M. (1980) Virology 106, 292.
14. DeFeo, D., Gonda, M.A., Young, H.A., Chang, E.H., Lowy, D.R., Scolnick, E.M. and Ellis, R.W. (1981) Proc. Natl. Acad. Sci. U.S.A. 78, 3328.
15. Ellis, R.W., DeFeo, D., Shih, T.Y., Gonda, M.A., Young, H.A., Tsuchida, N., Lowy, D.R. and Scolnick, E.M. (1981) Nature 292, 506.
16. Laemmli, U.K. (1970) Nature 227, 680.
17. Land, H., Parada, L.F. and Weinberg, R.A. (1983) Science 222, 771.
18. Cho-Chung, Y.S. (1979) Life Sci. 24, 1231.
19. Huang, F.L. and Cho-Chung, Y.S. (1983) Cancer Res. 43, 2138.
20. Sukumar, S., Notario, V., Martin-Zanca, D. and Barbacid, M. (1983) Nature 306, 658.