Neoplastic Transformation of Normal Rat Embryo Fibroblasts by a Mutated p53 and an Activated ras Oncogene Induces Parathyroid Hormone-related Peptide Gene Expression and Causes Hypercalcemia in Nude Mice*

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Parathyroid hormone-related peptide (PTHRP) is a 141-amino acid protein identified in various carcinomas associated with humoral hypercalcemia of malignancy (HHM). Although the causal role of PTHRP in HHM syndrome has been established, the molecular and cellular mechanism by which PTHRP gene is overexpressed in certain malignancies remains unknown. We have demonstrated in the present study that PTHRP secretion was markedly induced concomitantly with the formation of transformed foci after normal rat embryo fibroblasts (REFs) were co-transfected with an activated ras (ras) and a mutated form of p53 (p53-nt) genes. In either ras- or p53-nt-transfected (nontransformed) cells, only modest or barely detectable secretion of PTHRP was observed, respectively. Northern blot analysis revealed that PTHRP mRNA was markedly induced in fully transformed cells 11 days after transfection with both ras and p53-nt genes. Inhibition of RNA synthesis with actinomycin D resulted in almost complete disappearance of PTHRP mRNA at 2–3 h, suggesting a transcriptional mechanism. Transient transfection experiments revealed that PTHRP promoter activity was induced in ras + p53-nt transfectants. REFs transfected by ras and p53-nt genes and thereby induced to secrete PTHRP in vitro produced aggressively growing tumors associated with HHM syndrome when injected into nude mice. These results suggest that activation of PTHRP gene is closely related to malignant transformation of normal mammalian cells and that ras and p53 may be important regulators of PTHRP gene transcription. The transfection-focus formation system of REFs should provide an excellent model to study the molecular and cellular mechanism underlying concomitant overexpression of PTHRP gene with carcinogenesis.

Parathyroid hormone-related peptide (PTHRP)1 is a 141-amino acid protein identified in various carcinomas as a causative factor for humoral hypercalcemia of malignancy (HHM) (1–3). PTHRP shows structural and functional similarities to parathyroid hormone, including a sequence homology at the amino-terminal parathyroid hormone-like domain (1–3). Thus PTHRP overproduced and secreted by certain carcinomas enters the circulation (4), interacts with a common receptor for parathyroid hormone/PTHRP in bone and kidney (5), and causes hypercalcemia in cancer patients. The findings that circulating PTHRP levels are elevated in most patients with HHM (4) and that hypercalcemia is corrected by a neutralizing antibody against PTHRP in animal models of HHM (6) have further strengthened the causal role for PTHRP in the pathogenesis of HHM syndrome.

Accumulating evidence suggests that PTHRP gene is expressed at low levels in a number of normal tissues and plays diverse physiological roles mainly in an autocrine/paracrine fashion (7). However, the exact molecular and cellular mechanism by which PTHRP gene is overexpressed in certain carcinomas remains unknown.

Carcinogenesis is a complex, multistep process, and it has been established that oncogenes and tumor suppressor genes play the fundamental role as the genetic targets, the alteration of which defines each of the distinct steps in the multistep cellular transformation (8, 9). The complex process involving the conversion of normal cells to malignant phenotypes can be reconstructed in vitro using the transfection focus formation assay, which has been elaborated to test the oncogenic potentials of various tumor-related genes. Using this assay cytoplasmic oncogenes, represented by ras and src, and nuclear oncogenes, such as myc and mutated p53, have been shown to collaborate with each other to fully transform normal rodent cells by acting in a complementary way, culminating in tumor formation in vivo (10–12).

In the present study we have demonstrated that PTHRP gene expression is markedly induced in close association with transformation of normal rat embryo fibroblasts (REFs) by co-transfection of an activated ras gene and a mutated p53 gene. Furthermore, the transformed cells produced aggressively growing tumors with hypercalcemia in vivo when inoculated into nude mice. The current reconstruction system of HHM syndrome, starting with normal mammalian cells through the malignant transformation in vitro and tumor formation in vivo, may provide an excellent model to dissect the molecular and cellular events leading to the overexpression of metric assay; Ca²⁺, ionized calcium; CAT, chloramphenicol acetyltransferase.
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PTHRP gene in certain malignancies.

**MATERIALS AND METHODS**

Plasmids—pucEJ (13) (courtesy of Dr. Steven F. Dowdy, Whitehead Institute for Biomedical Research, Cambridge, MA) was used to express an oncogenic allele of Ha-ras (E) and, p53 KH215 (14) (courtesy of Dr. David V. Mangin, Yale University, New Haven, CT) and p53-ras (15) (courtesy of Dr. John J. Beauchamp, Boston, MA) with a DNA in-frame insertion of decameric HindIII linker into the KpnI site at amino acids 215–216 of the murine p53 sequence was used to express a mutated form of p53 (p53-mt). ras and p53 were chosen as target DNA since it has been demonstrated that these genes are mutated in a variety of human cancers and that these mutations cooperate with each other to convert normal fibroblasts to malignant phenotypes (12).

Cell Culture and Transfections—Rat embryo fibroblasts (REFs) were prepared from 14-day-old Wistar rat embryos (Tokyo Laboratory Animals Science Co. Ltd., Tokyo, Japan) as described previously (15), and grown for 2 days in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% fetal bovine serum and 60 μg/ml kanamycin. Adherent cells were trypsinized and frozen in liquid nitrogen until use.

For transfection experiments REFs were plated at 3–5 × 10^6/cm^2 and a total of 20 μg of plasmid DNA consisting of test plasmids or the control plasmid (psV-SPORT 1, Life Technologies, Inc.) and neo gene plasmid (pcDneo) was transfected on the following day by the calcium phosphate method according to Chen and Okayama (16). After 14–18 h, cells were washed with phosphate-buffered saline and cultured in fresh medium for an additional day. Then cells were trypsinized, replated into 10-cm dishes, and fed every 3–4 days thereafter. In order to monitor successful transfection, one-tenth of cells were trypsinized, replated into 10-cm dishes, and fed every 3–4 days thereafter. For transfection experiments one-tenth of cells were plated into a 6-cm dish and cultured in the presence of 400 μg/ml Geneticin (Gibco). The number of foci, the diameter of which was 3 mm or larger, was scored after Giemsa staining 11 days after the transfaction. At the indicated intervals, cells and the conditioned medium were harvested separately for RNA analysis and determination of PTHRP concentrations, respectively. Actinomycin D (Sigma) was dissolved in ethanol, and control cells received an equal volume of vehicle.

Animals—Five-week-old male athymic mice were purchased from Clea Japan Inc. (Tokyo, Japan) and maintained in sterilized cages, and fed standard diet containing 3.25% calcium and 1.06% phosphate (CE-2, Clea Japan Inc.). Tumorigenicity and the inducibility of hypercalcemia in vivo were studied by inoculating 5–10 × 10^5 cells subcutaneously into the right flank of nude mice. Tumor size was calculated by the following formula: tumor volume (m) = a × b × c/2, where a is the length (cm) and b is the width (cm) of the tumor. Blood was drawn every week by retroorbital plexus puncture for determination of blood ionized calcium (Ca^2+), ionized phosphate (P). Blood calcium concentrations were measured by the electrode method using an autoanalyzer (M-634, Chiba Corning Diagnostics Co. Ltd., Tokyo, Japan). At sacrifice blood was drawn by cardiac puncture for determination of plasma PTHRP concentrations. The animals were treated in accordance with Chugai Pharmaceutical's ethical guidelines of animal care, handling, and termination, and the current guidelines of animal care of the institution.

Immunoradiometric Assay (IRMA) for PTHRP—PTHRP concentrations were determined with a sensitive two-site IRMA for human PTHRP-(1–87) as described (17). In brief, conditioned medium or plasma (200 μl) was first incubated with 100 μl of affinity-purified anti-PTHRP (SD-83) polyclonal antibody and a polystyrene bead coupled to anti-PTHRP-(1–34) monoclonal antibody for 18 h. After washing with water, the bead was incubated with 125I-labeled anti-rabbit IgG goat antibody for additional 18 h, and radioactivity associated with the bead was counted after washing with water. The intraassay and interassay variations were 3.4–5.8% and 4.0–7.5%, respectively, and the detection limit, as determined by 2 standard deviations above the mean counts of nonspecific binding, was 0.5 pmol/liter (17).

Data were expressed as mean ± S.E., and statistical significance was determined by analysis of variance.

**RESULTS AND DISCUSSION**

In order to examine the relationship between malignant transformation and PTHRP overproduction, we first attempted to establish an in vitro system in which normal mammalian cells could be converted to transformed cells through introduction of known oncogenes. Normal rat embryo fibroblasts (REFs) were used in the transfection focus formation assay to test the oncogenic potentials of various tumor-related genes and shown to require at least two different types of oncogenes for full transformation (11, 12). As shown in Fig. 1, no foci of morphologically altered cells were seen in the control plasmid- or p53-mt-transfected cultures, and only small number of foci were seen 11 days after transfection of ras oncogene alone (4 foci/10-cm dish). In contrast, co-transfection with ras and p53-mt induced the appearance of large numbers of overgrowing foci of cells with transformed morphology 11 days after transfection (80 foci/10-cm dish). These results are in good agreement with the previous observations that an activated ras gene and a mutated p53 gene cooperate with each other in cellular transformation (12).

We next examined the expression of PTHRP gene in the transfection-focus formation system. The conditioned medium was harvested during the last 3 days, and PTHRP concentrations were determined by a sensitive and specific IRMA for human PTHRP-(1–87) (17) since no immunoassay specific to rat PTHRP was available. Amino acid sequences of PTHRP are remarkably conserved among various species up to the residue 111 (1), and it has been reported that IRMA for human PTHRP-(1–74) is capable of detecting circulating PTHRP derived from...
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The time course of the induction of PTHRP secretion is shown in Fig. 3. Cells co-transfected with ras and p53-mt genes секретed larger amounts of PTHRP, although not statistically significant. In contrast, fully transformed cells after co-transfection with ras and p53-mt genes secreted markedly elevated amounts of PTHRP per the same number of cells (Fig. 2). Dilution of the medium conditioned by ras + p53-mt transformants revealed a response curve parallel to that of human PTHRP-(1–87) standard in the IRMA, and immunoreactive PTHRP was barely detectable in plasma from non-tumor-bearing nude rats or mice (data not shown). These results suggest that the IRMA used in the current study cross-reacted with rat PTHRP, although we cannot rule out the possibility that our assay underestimated the concentrations of rat peptide.

The time course of the induction of PTHRP secretion after transfection of the oncogenes is shown in Fig. 3. Cells co-transfected with ras and p53-mt genes secreted gradually increasing but modest amounts of PTHRP until day 8 and were markedly induced to secrete large amounts of PTHRP between 8 and 11 days after transfection. Again, cells transfected with ras alone secreted much smaller amounts of PTHRP during the same period (Fig. 3). In good agreement with the results of Northern blot analysis performed using PTHRP and β-actin cDNA probes as described under “Materials and Methods,” the PTHRP gene has certain characteristics of an early response gene, including the presence in PTHRP mRNAs of the AUUUA instability motifs at the 3’-untranslated regions (25). In fact it has been demonstrated that PTHRP mRNA turns over rapidly in vitro (26) and in vivo (27) with the reported half-life ranging from 30 min to 2 h. In order to determine whether the induction of PTHRP mRNA in ras + p53-mt transfectedants occurred at a transcriptional or post-transcriptional level, the disappearance rate of PTHRP mRNA was assessed after replating the fully transformed cells. As shown in Fig. 4B, PTHRP mRNA level was markedly decreased at 2 h and disappeared at 3 h after the addition of actinomycin D, an inhibitor of RNA synthesis, suggesting that the expression of PTHRP gene was induced mainly at a transcriptional level. Furthermore, when transient transfection ex-
eminate and p53-mt genes (Fig. 5). The PTHRP promoter fragment used in the current transfection experiments contains binding sites for various transcription factors, including Sp 1, Ets, and AP-2 (28–30). Identification of a trans-acting factor as well as a DNA element responsible for PTHRP gene activation in ras + p53-mt-transformed cells will help gain further insight into the molecular mechanism underlying PTHRP overproduction in certain malignancies.

We finally examined whether cells fully transformed by ras and p53-mt genes and thereby produced large amounts of PTHRP in vitro were indeed capable of forming tumors and causing HHM syndrome in vivo. Nude mice were inoculated with ras + p53-mt transfectants and monitored for subsequent appearance of subcutaneous tumors and blood Ca²⁺ concentrations. As shown in Fig. 6 (left panel), animals inoculated with ras-transfected cells did not produce tumors or develop hypercalcemia, whereas those with the same number (1 × 10⁶) of cells co-transfected with ras and p53-mt genes produced aggressively growing tumors and developed hypercalcemia. The ability of ras + p53-mt transfectants to induce tumors associated with marked hypercalcemia in vivo was further confirmed in three out of four nude mice inoculated with 5 × 10⁶ cells in a separate experiment (Fig. 6, right panel). The fourth animal showed marginal hypercalcemia. As shown in Fig. 7A, peak values of blood Ca²⁺ were significantly higher in nude mice inoculated with ras + p53-mt transfectants than those in age-matched control animals, and the hypercalcemic nude mice displayed markedly elevated plasma PTHRP concentrations as compared with controls (0.6 ± 0.02 pmol/l) (Fig. 7B). Plasma PTHRP concentrations in these hypercalcemic animals were comparable with those in patients with HHM determined with the current IRMA (17). Evidently the tumors associated with hypercalcemia expressed high levels of a single transcript for rat PTHRP mRNA (Fig. 7C).

In summary, we have demonstrated in the present study that introduction of two types of oncogenes, EJ-ras and p53-mt, was necessary and sufficient not only for the conversion of normal mammalian cells into malignant cells but for the induction of PTHRP gene transcription in vitro and the development of HHM syndrome in vivo. A family of ras genes encode highly homologous proteins with molecular mass of 21 kDa, which play pivotal roles in the transduction of extracellular signals to the nucleus (31). It has been shown in a human keratinocyte model that a progressive dysregulation of PTHRP gene expression occurs during the conversion from established phenotypes conferred by human papilloma virus 16 to malignant transformation induced by ras oncogene (32). In addition, Li and Drucker (33) have recently reported that transfection of either EJ-Ha-ras or v-src oncogene into established cell lines, NRK 49F (fibroblasts) and RCB 2.2 (osteoblast-enriched cell line), induced PTHRP mRNA expression at a transcriptional level, although it remained to be proven whether the increased production of PTHRP after transfection with either EJ-ras or v-src alone was sufficient to cause HHM syndrome in vivo. We have observed in the current study that the transfection of EJ-ras alone into REFs resulted in a modest increase in

![Fig. 5. Induction of PTHRP promoter activity in transformed cells with ras and p53-mt.](image)

![Fig. 6. EJ-ras- and p53-mt-transformed cells induce hypercalcemia in nude mice.](image)

![Fig. 7. PTHRP expression in nude mice inoculated with ras + p53-mt transfectants.](image)
transfectants contained a modestly increased amount of p53-mt transfectants. Our preliminary analysis showed that cellular functions the second mutation confers upon transfection of human papilloma virus 16 DNA in the keratinocyte model. It remains to be elucidated, however, what alteration in mutation of p53 gene in the current system or the introduction of HHM syndrome in a relatively short period of time (11 days in vitro). Also, the association of HHM syndrome in esophageal carcinomas, and hepatocellular carcinoma (37). Although the biochemical activities of wild-type p53 and its mutations are not sufficient for full induction of PTHRP gene expression, and p53-mt, but not by transfection of p53-mt transfectants contained a modestly increased amount of p53-mt transfectants. Our preliminary analysis showed that cellular functions the second mutation confers upon transfection of human papilloma virus 16 DNA in the keratinocyte model. It remains to be elucidated, however, what alteration in mutation of p53 gene in the current system or the introduction of HHM syndrome in a relatively short period of time (11 days in vitro).

In our experimental system malignant transformation by both ras and p53-mt, but not by transfection of ras alone, was associated with a marked induction of PTHRP gene transcription in vitro and the reconstitution of HHM syndrome in vivo. Taken together, it is tempting to hypothesize that the development of HHM requires another genetic alteration, such as a mutation of p53 gene in the current system or the introduction of human papilloma virus 16 DNA in the keratinocyte model (32). It remains to be elucidated, however, what alteration in cellular functions the second mutation confers upon transformed cells. Our preliminary analysis showed that ras + p53-mt transfectants contained a modestly increased amount (1.5–2-fold) of ras mRNA compared with cells transfected with ras alone (not shown), which may suggest that the effect of a mutated p53 gene is mediated, at least in part, through the up-regulation of ras gene expression.

The p53 tumor-suppressor gene encodes a 53-kDa nuclear phosphoprotein involved in the control of cell proliferation (35, 36), and its mutations have been detected in approximately half of almost all types of human cancers, as diverse as colon and breast carcinomas, lymphomas and leukemias, lung and esophageal carcinomas, and hepatocellular carcinoma (37). Although the biochemical activities of wild-type p53 and its mutated forms are not entirely clear, there is accumulating evidence suggesting that p53 binds DNA specifically and affects the expression of various genes and that these functions of p53 are interfered with by its mutations (35, 36). The current system may be useful to examine the possible role for p53 mutation in the activation of PTHRP gene during the process of cellular transformation. Also, the association of HHM syndrome with the occurrence of p53 and ras mutations in human tumors remains to be elucidated.

In conclusion, the results of the present study suggest that the overproduction of PTHRP and development of HHM syndrome occur during the multistep transformation of normal mammalian cells to malignant phenotypes and that ras and p53 may be important upstream regulators of PTHRP gene transcription. Since our experimental system can reproduce HHM syndrome in a relatively short period of time (11 days in vitro and 4–5 weeks in vivo), it should provide an excellent model to further dissect the molecular and cellular events involved in the overexpression of PTHRP gene in certain malignancies.
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