Induction of Parathyroid Hormone-related Peptide by the Ras Oncogene: Role of Ras Farnesylation Inhibitors as Potential Therapeutic Agents for Hypercalcemia of Malignancy

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ABSTRACT

Parathyroid hormone related peptide (PTHRP) is the major causal agent in the syndrome of malignancy-associated hypercalcemia (MAH). Several studies have shown that PTHRP production is increased in response to growth factors and oncogenes, such as Tpr-Met, that are associated with the tyrosine kinase signaling pathway. Using site-directed mutagenesis of Tpr-Met and chemical inhibitors of phosphotidylinositol-3 kinase and Ras isoprenylation, we demonstrated previously that induction of PTHRP is mediated via the Ras signaling pathway. In the present study, we have directly investigated the role of the Ras oncogene in MAH. As a model system, we used Fisher rat 3T3 fibroblasts stably transfected with a Ras oncogene (Ras-3T3). Ras transfection enhanced PTHRP production 5-10-fold in these cells, and inoculation of this cell line into nude mice led to the development of hypercalcemia within 2 weeks. We used this system to evaluate the effect of a potent inhibitor of Ras processing, B-1086, on cell growth, PTHRP production, plasma calcium, and tumor growth. Treatment of Ras-3T3 cells in vitro with B-1086 at 0.1-10 μg/ml produced a significant reduction in PTHRP mRNA expression and PTHRP secretion and a significant decrease in cell proliferation. Treatment in vivo of BALB/c nu/nu mice bearing Ras-3T3 tumors with B-1086 resulted in a significant inhibition in tumor growth. In addition, this treatment produced near normalization of serum Ca++, a significant decrease in plasma PTHRP, and a reduction in tumoral PTHRP mRNA levels. These results show that the Ras pathway is involved in PTHRP production by tumors, identifies Ras as a potential target for treatment of MAH, and demonstrates Ras processing inhibitors as candidate therapeutic agents against this syndrome.

INTRODUCTION

Hypercalcemia of malignancy is a major complication in a significant portion of cancer patients and PTHRP has been identified as the major pathogenic factor for this disease (1–5). PTHRP shares a high degree of homology in its NH2-terminal region with PTH and because of this sequence homology is able to mimic the actions of PTH by interacting with a common PTH/PTHRP receptor (6, 7). In normal physiology, PTHRP is widely expressed, acts in an autocrine or paracrine manner, and has been shown to have a broad spectrum of functions especially related to modulating cell growth and differentiation (8–11). PTHRP appears to be a key regulator of skeletal development in the fetus but has also been implicated in transepithelial calcium transport in such sites as mammary epithelium, kidney, and placenta (12–16). Furthermore, PTHRP has been shown to be a potent muscle relaxant acting on the smooth muscle of the uterus, urinary bladder, and vascular wall (17). In malignancy-associated hypercalcemia, PTHRP acts primarily as an endocrine factor (18). When large quantities of PTHRP are produced by cancer cells, PTHRP is secreted into the circulation and acts at the level of bone to stimulate bone resorption and Ca++ release and on the kidneys to increase Ca++ reabsorption (19). The outcome of these actions is the development of hypercalcemia, which may be fatal. Increasing number of studies on the regulation of PTHRP gene expression are beginning to shed light on the molecular mechanisms responsible for PTHRP overproduction by cancer (20, 21). We have previously shown the critical role played by growth factors, acting on tyrosine kinase receptors, in stimulating PTHRP production (22). We subsequently demonstrated the effect of the oncogenic derivative of the hepatocyte growth factor/scatter factor receptor, Tpr-Met, on increasing PTHRP production and demonstrated that the mechanism of this stimulation involves the intracellular Ras signaling pathway (22). Previous studies by us and others, examining the relationship between tyrosine kinases and PTHRP, have indeed indicated that Ras is at the cross-roads through which the signal for PTHRP induction is propagated. Ras genes play a fundamental role in basic cellular functions (23). Ras proteins are GDP/GTP-regulated switches that in their mature functional form are anchored to the cell membrane by an isoprenyl tail (24, 25). In normal physiology, Ras functions in transducing developmental and proliferative signals from the cell surface to the nucleus. In addition to its role in growth and differentiation, Ras is also important in controlling cytoskeletal protein expression and in regulating vesicle trafficking within the cell (26). Perhaps owing to its central role within the cell, alteration of this gene is often associated with the initiating steps that lead toward carcinogenesis. Indeed, activating mutations in the Ras gene account for at least 40% of human neoplasia and play a significant role in progression of these malignancies (27). In light of this, Ras is an ideal target for therapeutic intervention to combat both the establishment of the primary cancer and for the prevention of secondary syndromes (28). One approach to target Ras has been the development of peptidomimetic inhibitors of Ras, which mimic the farnesylatation site of the Ras protein, a CAAX consensus sequence at the COOH-terminal, and act by competitively inhibiting Ras farnesylation (29, 30). Farnesylation is essential for subsequent modifications of Ras, for membrane localization, and ultimately for functioning of this oncogenic protein (31).

In the present study, using Fisher rat 3T3 fibroblasts transfected with the Ras oncogene, we have examined the role of Ras in PTHRP production and in the development of hypercalcemia in vivo. We have also tested the ability of a Ras farnesyl transferase inhibitor B-1086 to alter the course of the tumor and its production of PTHRP and assessed the effects on the hypercalcemic syndrome in vivo.

MATERIALS AND METHODS

Ras Farnesyl Transferase Inhibitor B-1086. The Ras farnesyl transferase inhibitor, B-1086 (29), was kindly provided by Dr. Michael Lewis of Eisai Research Institute (Andover, MA).

Animal Protocol. Female BALB/c nu/nu mice (Charles River Breeding Laboratories, Wilmington, MA), weighing 15–20 g (4–5 weeks of age) were injected on day 0 s.c. into the left and right flanks with Ras-3T3 cells (1 × 105) suspended in 0.1 ml of sterile saline. Animals were housed individually and fed...
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**ad libitum** on autoclaved standard rodent chow (rat chow 5012;Ralston-Purina Canada, Inc., Lasalle, Quebec, Canada) containing 1% calcium and 0.74% phosphorus and sterilized tap water (32). On day 5, vehicle, 50 mg/kg B-1086, or 100 mg/kg B-1086 was administered daily i.p. into anesthetized animals. All animals were examined for the development of tumors daily for up to 15 days. On the day of sacrifice, animals were bled by intracardiac puncture, and tumor volume of control and experimental animals was measured in two dimensions by calipers, and the tumor volume was calculated {[(width + length)/2]}^3. On the day of sacrifice, animals were bled by intracardiac puncture, and tumor tissue was extracted and immediately frozen in an ethanol bath for RNA analysis.

**Cells and Cell Growth.** The Fisher rat (Fr) 3T3 fibroblasts expressing the Ras oncogene were generated by ecotropic retroviral infection as described previously (22). Transient transfection into COS-1 cells of the pLXS2 retroviral vector encoding the Ras gene and G418 resistance and of the pSV-Y-EMLV plasmid encoding retroviral packaging proteins was performed by the DEAE-dextran method. At 12 h after transfection, cells were treated with 100 mm chloroquine for 3 h and incubated for an additional 48–72 h before harvesting. Transfected COS-1 cells were lysed, and the supernatant containing the packaging virus, at a titer of 5 × 10^5 plaque-forming units, was used to infect Fr 3T3 cells. Experimental cell lines were then selected for G418 resistance and foci formation.

For growth curves, Fr 3T3 cells were plated in medium containing 2% fetal bovine serum in 9.6-cm² Petri dishes (Falcon) at seeding densities of 5 × 10^3 cells/plate. Cells from replicate dishes were trypsinized and counted, daily, in a model Z Coulter counter (Coulter Electronics, Hialeah, FL). Medium was changed in all plates every 24 h (22).

**Northern Blot Analysis.** Total cellular RNA was isolated from the control and experimental cells, following treatment with vehicle alone or graded concentration of B-1086, by acid guanidinium thiocyanate-phenol-chloroform extraction as described previously (22). Twenty µg of total cellular RNA were electrophoresed on a 1.1% agarose-formaldehyde gel, transferred to nitrocellulose membranes, and immunoblotted with anti-ras antibody (Santa Cruz Biotechnology). The signals were visualized using chemiluminescence (Amersham Corp.).

**Ras Processing Assay.** Fr-3T3 cells were plated in medium containing 10% calf serum, and 24 h later, treated with 0, 10, or 50 µg/ml of B-1086 for 72 h and harvested in lysis buffer (PBS, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 10 µl/ml PMSF, 30 µl/ml aprotinin, and 10 µl/ml sodium orthovanadate). The lysates (25 µg) were electrophoresed on a 15% SDS-polyacrylamide gel, transferred to nitrocellulose membranes, and immunoblotted with anti-ras antibody (Santa Cruz Biotechnology). The signals were visualized using chemiluminescence (Amersham Corp.).

**Other Analytical Methods.** For RIA, conditioned medium (2 ml/well of a six-well cluster plate) was removed from control and experimental 3T3 cells treated with vehicle alone or with graded doses of B-1086 for 24 h. Duplicate 200–500-µl aliquots of cell-conditioned culture medium were evaporated to dryness in a Speedvac (Savant Instruments, Inc., Hicksville, NY) and stored at −20°C until assayed. Dried medium was reconstituted with 200 µl of outdated blood bank plasma and radioimmunoassayed as described previously using ^125_labeled [Tyr] PTHRP(1–34) as a tracer and PTHRP(1–34) as a standard (34). Results are expressed as nanogram equivalents of PTHRP(1–34)/10^6 cells.

Fig. 1. Effect of Ras expression on PTHRP production. A. Northern blot analysis of Fr 3T3 cells infected with empty vector alone (V) or with experimental vector (Ras). Cells were grown in the absence of serum, and 20 µg of total cellular RNA were extracted and electrophoresed on a 1.1% agarose-formaldehyde gel. Filters were probed with a ^32P_labeled PTHRP cDNA or with a ^32P_labeled 18S RNA probe as described in Materials and Methods. All filters were quantitated by densiometric scanning (lower panel). B, conditioned culture medium from Fr 3T3 cells infected with empty vector alone (V) or with experimental vector (Ras) was collected after 48 h and analyzed for PTHRP immunoreactivity (iPTHRP) as described in Materials and Methods. Results are expressed as ng equivalents of PTHRP(1–34) (ng eq. 1–34). Significant differences from control cells are represented by asterisks. * P < 0.05. Bars, SE.
RESULTS

Effect of the Ras Oncogene on PTHRP Production. To examine the effect of Ras oncogene transfection on PTHRP production, total cellular RNA was extracted from control and Ras-transfected cells and analyzed by Northern blot analysis. Both control and experimental cell lines were maintained in culture for at least 24 h in the absence of serum to rule out any change in PTHRP expression by serum-derived growth factors. Transfection of the Ras oncogene caused a marked increase (5-10-fold) in PTHRP mRNA expression (Fig. 1A). The effect of transfection of this oncogene on PTHRP secretion into the cell-conditioned culture medium was determined by RIA, and comparison was made with the amount of PTHRP released into the wild-type and vector-transfected 3T3 cells. Secretion of immunoreactive PTHRP was significantly greater in 3T3 cells transfected with Ras as compared to control cells (Fig. 1B). These results demonstrated that Ras itself was a potent inducer of PTHRP production, independent of any growth factors present in the cell culture medium.

Effect of B-1086 on Ras Processing and PTHRP Production by Ras-3T3 Cells in Vitro. To examine the capacity of B-1086 to inhibit Ras processing, Ras 3T3 cells were treated with B-1086 (10 or 50 µg/ml). B-1086 produced a dose-dependent reduction in Ras protein processing in Ras-3T3 cells (Fig. 2). Treatment with vehicle alone (0.1% DMSO) revealed no inhibitory effect on Ras processing.

The ability of B-1086 to influence PTHRP production was examined in experimental Ras-3T3 cells in vitro. Ras-3T3 cells were treated with increasing concentrations (0.01–10 µg/ml) of B-1086 for 48 h. At the end of this treatment, cells were isolated, and total cellular RNA was extracted and analyzed for PTHRP mRNA expression by Northern blot analysis. Conditioned medium from control and experimental cells was assayed for immunoreactive PTHRP by an NH₂-terminal PTHRP RIA. B-1086 caused a dose-dependent decrease in PTHRP mRNA expression (Fig. 3A) and secretion of PTHRP protein into conditioned culture medium (Fig. 3B). Morphological analysis of Ras-3T3 cells following treatment with graded doses of B-1086 and cell viability assay using trypan blue failed to show any cytotoxic effects of B-1086 in concentrations used in this study (data not shown).

Effect of B-1086 on the Growth of Ras-3T3 Cells. The effect of cell growth following transfection with the Ras oncogene was examined for 6 days in vitro, and comparison was made with the growth of wild-type cells and with Ras-3T3 cells treated with B-1086 (10 µg/ml). Transfection of the Ras oncogene caused a significant increase in cell growth as compared to wild-type cells or cells transfected with vector alone (data not shown). Treatment of Ras-3T3 cells

Fig. 2. Treatment with B-1086 inhibits Ras protein processing in Ras-3T3 cells in vitro. Ras-3T3 cells were grown to 70% confluence and treated with either vehicle, 10 µg/ml of B-1086, or 50 µg/ml of B-1086 for 72 h. The cell lysates were electrophoresed and immunoblotted with anti-Ras antibody and analyzed for Ras processing as described in "Materials and Methods." U, unprocessed; P, processed forms of Ras.

Fig. 3. Effect of B-1086 on PTHRP production in Ras-3T3 cells. A. Fr 3T3 cells transfected with the empty vector (V) or with the experimental plasmid (Ras) were grown to 70% confluence. Ras-transfected cells were incubated with 0, 0.1, or 10 µg/ml B-1086. Twenty µg of total cellular RNA were extracted from control and experimental cells and electrophoresed on a 1.1% agarose formaldehyde gel. Filters were probed with a 32P-labeled PTHRP cDNA or with a 32P-labeled 18 S RNA probe as described in "Materials and Methods." All blots were quantified by densitometric scanning (lower panel). B. Conditioned culture medium from control Fr 3T3 cells (V) and from Ras-transfected Fr 3T3 cells treated with graded concentrations of B-1086 was collected after 48 h and analyzed for PTHRP immunoreactivity as described in "Materials and Methods." Significant differences from control cells are represented by asterisks. Bars, SE. *P < 0.05.
crease in their plasma PTHRP levels (Fig. 1B).

day 15, blood was collected and analyzed for plasma PTHRP. Treatment of animals with B-1086 (100 mg/kg) caused a significant decrease in plasma PTHRP levels (Fig. 2). Additionally, treatment of animals with B-1086 (50 or 100 mg/kg) for 10 days (day 6-15) resulted in a significant reduction in tumor volume as compared to animals infused with vehicle alone, and the effects of B-1086 on decreasing tumor volume were dose dependent (Fig. 5).

Effect of B-1086 on Serum Ca^{2+} of Ras-3T3 Tumor-bearing Mice. Ras-3T3 cells were inoculated into the right flank of nude mice, and animals were monitored for the development of hypercalcemia. Inoculation of Ras-3T3 cells resulted in the development of palpable tumors by 6 days and hypercalcemia by day 15 after tumor cell inoculation. Treatment of these experimental animals with B-1086 (100 mg/kg) for 10 days (day 6–15) resulted in a significant reduction in their serum Ca^{2+} levels, which appeared to be dose dependent (Fig. 6).

Effect of B-1086 on Tumoral PTHRP Production in Vivo. On day 15 after tumor cell inoculation, all control and experimental animals were sacrificed, and their tumors were removed to analyze the level of tumoral PTHRP mRNA expression. Treatment of experimental animals with B-1086 (100 mg/kg) caused a significant decrease in PTHRP mRNA expression (Fig. 7A). Also at the time of sacrifice, on day 15, blood was collected and analyzed for plasma PTHRP. Treatment of tumor-bearing mice with B-1086 caused a significant decrease in their plasma PTHRP levels (Fig. 7B).

DISCUSSION

In previous studies, we demonstrated that PTHRP secretion by a tumor cell model is largely constitutive (32). Consequently, in contrast to PTH, the secretion of which is tightly regulated by concentrations of the extracellular calcium ion such that calcimimetics acting on the calcium receptor can block PTH secretion, it appeared unlikely that attempts to reduce PTHRP release by inhibition of secretion would be rewarding (36). Indeed, the rate of PTHRP secretion appears to be a direct function of its rate of synthesis, and the capacity of a given tumor to release PTHRP has been found to correlate with its level of gene expression (37, 38). Consequently, we have used alternative strategies to inhibit PTH release in vitro and to test these effects on in vivo models.

One approach we used previously emanated from our in vitro observations that PTHRP gene transcription could be inhibited by the active metabolite of vitamin D, 1,25-dihydroxyvitamin D (11, 39). Indeed, 1,25-dihydroxyvitamin D and a nonhypercalcemic analogue were then shown to diminish PTHRP production by tumors in vivo (40). A second approach we have used was to employ antisense technology to diminish PTHRP translation in vitro, and this strategy also reduced PTHRP production by tumors in vivo (41). Finally, a third strategy was to inhibit the posttranslational processing of PTHRP. PTHRP is synthesized as a prohormone, which has low intrinsic bioactivity; when the prohormone sequence is removed, the mature form is a fully active entity. We demonstrated that this cleavage can be accomplished by a furin-like prohormone convertase (42) and then showed that inhibition of the action of furin in tumors that overproduce PTHRP reduces levels of bioactive PTHRP in vitro and is also effective in vivo (41, 42). Consequently, several approaches may be used based on mechanisms of PTHRP regulation that have been determined in vitro, which can diminish PTHRP production by tumors implanted in vivo.

The use of Ras farnesylation inhibitors to inhibit PTHRP production was based on our initial observations that growth factors such as epidermal growth factor or insulin-like growth factor I can be potent stimulators of PTHRP gene transcription in vitro (11, 14, 37). Subsequently, we reported that an oncogenic, constitutively active, growth factor receptor, Tpr-Met, could also increase PTHRP produc-

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tion and that the Ras signaling pathway was involved in this stimulation. In the present study, we have shown that the Ras oncogene itself is a potent stimulus for PTHRP production in vitro and that overexpression of Ras in tumors in vitro can also lead to augmented PTHRP release and to the development of hypercalcemia. Furthermore, a potent inhibitor of Ras action can reverse these effects both in vitro and in vivo.

The cascade leading to increased PTHRP production involves binding of growth factors such as epidermal growth factor or hepatocyte growth factor to their receptors, activation of Ras proteins by exchange of bound GDP for GTP, and the triggering of a phosphorylation cascade that involves Raf kinase and mitogen-activated protein kinase; the end result is the activation of nuclear transcription factors that can apparently enhance PTHRP gene transcription and that can also regulate cell growth (43–45).

Signal transduction by Ras is dependent on its capacity to localize to the plasma membrane which, in turn, is dependent on farnesylation of the cysteine in the CAAX consensus sequence at the COOH terminus of the Ras protein (31). Inhibition of farnesylation is, therefore, being explored by several laboratories as a potential mechanism for inhibiting tumor growth. In the present study, we used a small organic molecule that mimics the tetrapeptide farnesylation site of Ras protein but is a methyl ester prodrug. It is more potent than the free acid form of the drug (B956) in vitro, because of its higher membrane permeability, but in vivo is rapidly hydrolyzed to the acid within the circulation (29). This inhibitor, in common with other farnesyl transferase inhibitors (29–31), reduced Ras posttranslational processing and also decreased cell growth. Furthermore, infusion of different concentrations of B-1086 used in this study failed to show any evidence of toxicity or change in the body weight of experimental animals as compared to control animals receiving vehicle alone (29).

The Ras oncogene is the site of one of the most prevalent genetic alterations in human cancer and can occur in a broad spectrum of cancers, many of which have been demonstrated to overproduce PTHRP (20, 21). Consequently, inhibition of Ras action appeared to be a particularly effective site to target to inhibit PTHRP production and the hypercalcemic syndrome as well as to reduce tumor growth. Nevertheless, human cancers evolve as a result of multiple genetic alterations, and inhibition of more than one disordered function may be needed to retard tumor progression. Therefore, with increased knowledge of the control of PTHRP production, targeting the multiple sites of aberrant regulation may be the most effective means of reversing increased secretion of this protein. Inhibition of Ras action should, nevertheless, be an important component of this approach.
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