Clinical Endocrinology (2000) **53,** 621–628

Parathyroid hormone-related protein in the aetiology of fibrous dysplasia of bone in the McCune Albright syndrome

William D. Fraser*, Cathy A. Walsh‡, Mark A. Birch§, Brian Durham*, Jane P. Dillon†, David McCreavy* and James A. Gallagher†

**University Department of Clinical Chemistry, Royal Liverpool University Hospital,* †*Human Bone Cell Research Group, Department of Human Anatomy and Cell Biology, The University of Liverpool,* ‡*Human and Applied Biology, Liverpool Hope University College, Hope Park, Liverpool,* §*Department of Trauma & Orthopaedic Surgery, The Medical School, University of Newcastle, Newcastle, UK*

(Received 17 February 2000; returned for revision 23 March 2000; finally revised 15 May 2000; accepted 30 June 2000)

Summary

OBJECTIVE Fibrous dysplasia, observed in bone lesions in the McCune Albright syndrome (MAS), is thought to result from abnormalities in cells of the osteogenic lineage associated with over-activation of the cAMP signalling pathway in affected cells. The aim of this study was to investigate the role of parathyroid hormone-related protein (PTHrP) in the aetiology of MAS, and to determine a possible therapeutic role for 1,25-dihydroxy vitamin D_3 ($1,25(OH)_2D_3$).

DESIGN The effects of $1,25(OH)_2D_3$ on PTHrP production and mRNA expression were determined *in vitro*. $1,25(OH)_2D_3$ therapy was administered to three patients with MAS.

PATIENTS Clinical data from four MAS patients (MAS1, 2, 3 and 4), and *in vitro* studies using bone from three MAS patients (MAS1, 2, and 3), are presented.

MEASUREMENTS Immunoradiometric assay and low-cycle number reverse transcriptase-linked PCR were used to determine PTHrP production and mRNA expression *in vitro*. Standard clinical biochemistry was recorded pre and post commencement of $1,25(OH)_2D_3$ treatment.

RESULTS We report the elevated secretion of PTHrP, and a concomitant rise in PTHrP mRNA expression, in cultured osteoblasts from three MAS patients. Treatment with $1,25(OH)_2D_3$ produced a dose-dependent decrease in PTHrP protein secretion and mRNA expression. Marked improvement in bone biochemistry in MAS1, 2 and 3 post treatment with $1,25(OH)_2$ D_3 is documented.

CONCLUSION This study provides the first evidence suggesting that PTHrP may contribute to the aetiology of fibrous dysplasia in MAS. In addition, the therapeutic administration of $1,25(OH)_2D_3$ may provide clinicians with an important new regime for symptomatic relief of bone pain and fracture in some patients with MAS.

The McCune Albright syndrome (MAS) is a sporadic, noninherited disorder characterized by polyostotic fibrous dysplasia, café au lait skin lesions and endocrine dysfunction (McCune, 1936; Albright *et al*., 1937; McCune & Bruch, 1937; Benedict, 1962; Mauras & Blizzard, 1986). This disorder arises from a dominant somatic mutation in early development resulting in a mosaic population of cells with and without the mutation (Happle, 1986). A single base change from arginine^{201} to cysteine or histidine leads to an abnormal G_s protein, with a resultant constitutive over-activation of the cAMP signalling pathway in affected tissues, including bone (Landis *et al*., 1989; Schwindinger *et al*., 1992; Shenker *et al*., 1994; Weinstein *et al*., 1999).

Histological examination of bone from patients with MAS reveals the presence of spindle-like fibrous cells in the bone marrow, and a haphazard organization of trabeculae associated with random orientation of collagen fibres and focal osteomalacia (Shenker *et al*., 1994). In addition, osteoblasts involved in the formation of bone lesions in MAS produce a matrix, which is enriched in versican and osteonectin, but poor in the adhesion molecules osteopontin and bone sialoprotein (Riminucci *et al*., 1997).

It is becoming increasing apparent that the fibrous dysplasia associated with bone lesions in MAS results from a dysfunction in cells of the osteogenic lineage (Marie *et al*., 1997; Riminucci *et al*., 1997). Changes in osteoblastic proliferation and differentiation resulting from over-activation of cAMP pathways have been reported (Marie *et al*., 1997).

Correspondence: Dr C. A. Walsh, Human and Applied Biology, Liverpool Hope University College, Liverpool L16 9JD, UK.
E-mail: walshc@hope.ac.uk

In this study, we have investigated the role of parathyroid hormone-related protein (PTHrP) in the aetiology of fibrous dysplasia in MAS. Human osteoblasts express PTHrP mRNA and produce PTHrP *in vitro* (Walsh *et al*., 1994, 1995). The protein is thought to be an important intracrine/paracrine/autocrine regulator of osteoblast function. As PTHrP secretion is regulated in part by cAMP (Inoue *et al*., 1993), production of the protein could be significantly altered by G-protein mutations. We report here that the secretion of PTHrP and expression of PTHrP mRNA is significantly elevated in cells derived from the bone of patients with MAS. The reduction of both mRNA and protein production in osteoblasts after $1,25(OH)_2D_3$ treatment observed *in vitro* was used to clinical benefit in three patients with MAS.

Methods

Clinical details

Clinical details of four MAS patients are presented in Table 1. In addition, trabecular bone was obtained from rib or vertebral body of control subjects undergoing corrective surgery, four age-matched to MAS1 (mean age 26·8 years, SD 2·8), and 15 age-matched to MAS2 and MAS3 (mean age 11·1 years, SD 4).

These studies were undertaken after obtaining approval of the hospital ethics committee and informed patient or parental consent.

Culture of human osteoblast

Human osteoblasts were derived from fragments of bone as described (Gallagher *et al*., 1996). Briefly, fragments of bone were explanted into culture in Dulbecco's Modified Eagle Medium supplemented with 10% foetal calf serum, L-glutamine and antibiotics (all Sigma Chemical Company, Poole, UK). At confluence, cells were treated for 48 h with $1,25(OH)_2D_3$ over the range 0·04–400 μg/l (Calbiochem, Nottingham, UK).

Assays

After incubation, conditioned media and cell layer proteins were collected. Immunoreactive PTHrP was measured using an immunoradiometric assay (IRMA) (Nichols Institute, San Juan Capistrano, CA, USA) (Fraser et al., 1993) and values expressed per mg of cell layer protein (Walsh et al., 1994). Cell layer protein was determined using a commercially available kit (MicroLowry, Sigma Chemical Company).

Molecular studies

mRNA was isolated from confluent monolayers of osteoblasts by MicroFastrack kit (Invitrogen, Carlsbad, CA, USA). Reverse transcriptase-linked polymerase chain reaction (RT-PCR) for PTHrP, and the constitutively expressed gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was undertaken as previously reported (Bilbe *et al*., 1996). PCR products were

Table 1 Clinical details of the four MAS patients studied

Patient code	Age at commencement of study (years)	Gender	Clinical signs and symptoms prior to study	Surgical therapies
MAS1	31	Female	Café au lait pigmentation, precocious puberty, severe menorrhagia, short stature, bone deformities, focal osteomalacia, haphazard trabecular organization	Hysterectomy, bilateral oophorectomy, multiple osteotomies
MAS2	13	Female	Café au lait pigmentation, endocrine abnormalities, short stature, bone deformities, focal osteomalacia, haphazard trabecular organization	Multiple osteotomies
MAS3	13	Male	Café au lait pigmentation, endocrine abnormalities, short stature, bone deformities, focal osteomalacia, haphazard trabecular organization	Multiple osteotomies
MAS4	18	Female	Endocrine abnormalities, short stature, bone deformities, focal osteomalacia, haphazard trabecular organization	Multiple osteotomies

analysed by agarose gel electrophoresis and Southern blotting (Walsh *et al*., 1994).

Genomic DNA isolation

Genomic DNA, isolated from bone (500–750 mg of tissue) and osteoblasts (10^6 cells) of three MAS patients using a commercial kit (Invitrogen), was used as a template in a PCR reaction. Oligonucleotide primers, 5′TCGGGTTGGCTTT GGTGAATCCA3′ and 5′AGAAACCATGATCTCTGTTATA3′ designed from introns 7 and 8 of the G_s gene were used to prime a PCR cycle of: 94 °C for 5 minutes followed by 35 cycles of 94 °C for 30 s; 52 °C for 30 s; 72 °C for 30 s and an elongation step of 5 minutes at 72 °C, which amplified a 163 base pair product. PCR products were run on agarose gels, cleaned using Qiaquick columns (Qiagen, Crawley, UK) and sequenced by automated sequencing.

Immunohistochemical detection of PTHrP

Tissue was fixed in formol saline for 24 h, decalcified in buffered 12% EDTA and paraffin embedded. Five micrometre sections were mounted onto Vectabond (Vector Labs, Southgate, UK) treated slides and dried overnight in a 50 °C oven before dewaxing with xylene. Endogenous peroxide activity was inhibited by preincubation in 1% H_2O_2 in phosphate buffered saline for 30 minutes, whilst nonspecific staining was blocked by a 30 minute incubation in normal swine serum. Sections were then stained with a 1 : 200 dilution of primary rabbit antiserum raised against human PTHrP(43–52). Sections were incubated with a 1 : 500 dilution of HRP-conjugated secondary antibody, goat antirabbit IgG whole molecule (Dalco, Cambridge, UK), for 60 minutes. The sections were then treated with Sigma fast 3,3′diaminobenzidine (DAB) tablets according to the instructions of the manufacturer and counterstained with Meyer's haemotoxylin.

Statistical analysis

ANOVA, followed by Duncan's new multiple range test, was carried out using a commercially available package (SPSS, Woking, UK).

Results

Concentrations of immunoreactive PTHrP, present in conditioned medium from confluent monolayers of osteoblasts, were quantified by IRMA (Table 2). Secretion of PTHrP by osteoblasts derived from MAS1 remained high over a 6-month culture period (data not shown) and medium conditioned by these cells diluted in parallel to the PTHrP standard curve confirming the identity of the protein (Table 3, Fig. 1).

Table 2 PTHrP production by osteoblasts derived from the bone of MAS1, MAS2 and MAS3 and age-matched controls. Medium, conditioned for 72 h by confluent monolayers of cells, was collected and PTHrP production determined by IRMA

Sample	PTHrP production (ng/l)
MAS1	4400
MAS2	1150
MAS3	8400
Age-matched to MAS1*	375 ± 5
Age-matched to MAS2/MAS3†	251 ± 5

* Mean ±SD PTHrP production ($n = 5$); †Mean ±SD PTHrP production ($n = 15$).

Molecular studies

RT-PCR. Figure 2 shows RT-PCR amplification of mRNA obtained from a 17-year-old female control subject and MAS1. A 535-bp product was clearly demonstrated after 23 thermal cycles using mRNA from MAS1 as template. However, a signal could not be detected after 23 cycles in mRNA from the control subject. Signals could not be detected after 18 cycles in either sample. GAPDH signals were of equal intensity in control and MAS mRNA.

Treatment of osteoblasts from MAS1 with doses of $1,25(OH)_2D_3$ from 0·04 μg/l to 400 μg/l produced a statistically significant ($P < 0{\cdot}01$), dose dependent decrease in PTHrP secretion (Fig. 3). There was a concomitant decrease in the steady state levels of PTHrP mRNA expression in cultures treated with 400 μg/l $1,25(OH)_2D_3$ which is seen clearly at 23 cycles and confirmed by Southern blot analysis at both 18 and 23 cycle products (Fig. 4). Steady state levels of GAPDH mRNA expression were unaffected by $1,25(OH)_2D_3$ treatment.

Table 3 Medium, conditioned for 72 h by a confluent monolayer of MAS1 cells, was serially diluted over the range 1: 4–1: 64. Immunoreactive PTHrP was measured by IRMA

Dilution of conditioned medium	Measured concentration (pmol/l)	Estimated concentration (pmol/l)	% of measurable baseline concentration
Undiluted	> 50·0	> 50·0	
× 2	> 50·0	> 100·0	
× 4	30·6	122·4	100
× 8	15·2	121·6	99
× 16	7·9	126·4	103
× 32	4·7	150·4	122
× 64	2·2	140·8	115

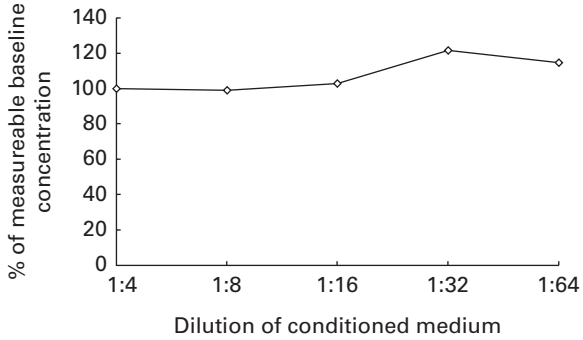

Fig. 1 Medium, conditioned for 72 h by osteoblasts derived from MAS1, was diluted with assay buffer. A profile of percentage of measurable baseline PTHrP concentration against increasing medium dilution is shown.

Genomic DNA. In genomic DNA isolated directly from bone the arginine[201] to cysteine mutation was detected in MAS1 and MAS2, and the arginine[201] to histidine mutation in MAS3. In genomic DNA isolated from osteoblasts, detection of mutations in the Gs gene was variable, yielding a low or negative signal. When present the mutation detected in human bone cells confirmed the base change detected in bone samples. These results indicated that the cultures contained a mosaic population of cells, the majority being wild type with only a small population of cells carrying the Gs mutation.

Immunohistochemical studies

Sections of bone from MAS patients were stained using a dilution of rabbit anti-human PTHrP (43–52) antiserum (IDS Ltd, Bolton, UK). Staining was observed in cells of the osteogenic lineage, including osteoblasts and osteocytes (Fig. 5).

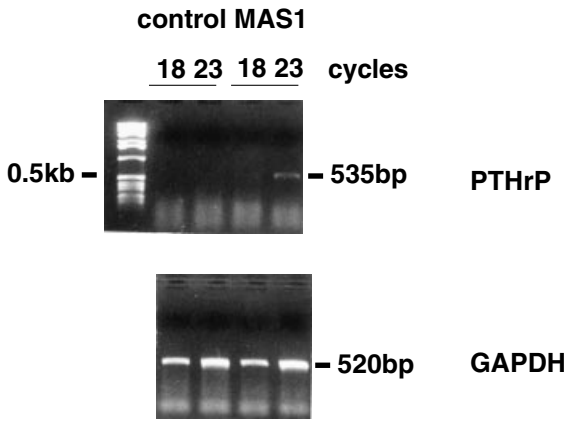

Fig. 2 RT-PCR amplification products obtained at 18 and 23 thermal cycles from cultured osteoblasts of a control subject and MAS1. The 535 bp product represents PTHrP transcripts and GAPDH is included as a constitutive control to verify the integrity of the cDNA.

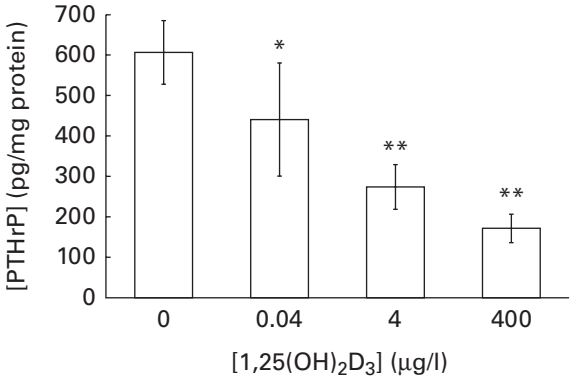

Fig. 3 Osteoblasts derived from the bone of a patient with MAS were treated in culture for 48 h with $1,25(OH)_2D_3$ using doses ranging from 0·04 μg/l to 400 μg/l. Values shown are mean PTHrP produced SD (pg/mg cell layer protein) ($n = 6$). $^*P < 0{\cdot}05$, $^{**}P < 0{\cdot}001$.

Clinical studies

When data from the molecular studies became available, a trial period of treatment of three patients (MAS1, MAS2 and MAS4) with 500 ng of $1,25(OH)_2D_3$ daily was begun. MAS3 was not given $1,25(OH)_2D_3$ as this patient had a history of renal stone formation when previously given calcium supplements and a plasma adjusted calcium (ACa) at the upper limit of the reference range (Table 4). The biochemical results of interest are given (Table 4). Following treatment with $1,25(OH)_2D_3$, alkaline phosphatase concentration in MAS1 fell to the lowest level recorded. Clinically there was a marked symptomatic improvement in MAS1, MAS2 and MAS4 with reduction in bone pain and a significant reduction in the number of fractures noted during the period of $1,25(OH)_2D_3$ treatment. Urinary calcium excretion increased following treatment in MAS1 and MAS2 from 176 to 296 and 120–184 mg/day, respectively (reference range 100–300 mg/day). Fourteen months and 20 months following commencement of this treatment MAS1 required further corrective surgery on both lower limbs. Bone samples were obtained for culture. PTHrP production by osteoblasts derived from the bone after commencement of $1,25(OH)_2D_3$ treatment was significantly decreased when compared to PTHrP production by osteoblasts derived from bone prior to treatment. PTHrP production was 4400, 15 and 103 ng/l from bone pretreatment, 8 and 14 months after commencement of $1,25(OH)_2D_3$ treatment, respectively.

Discussion

PTHrP was originally identified as a mediator of hypercalcaemia of malignancy. It has subsequently been shown to play an important role in the normal growth and development of a range

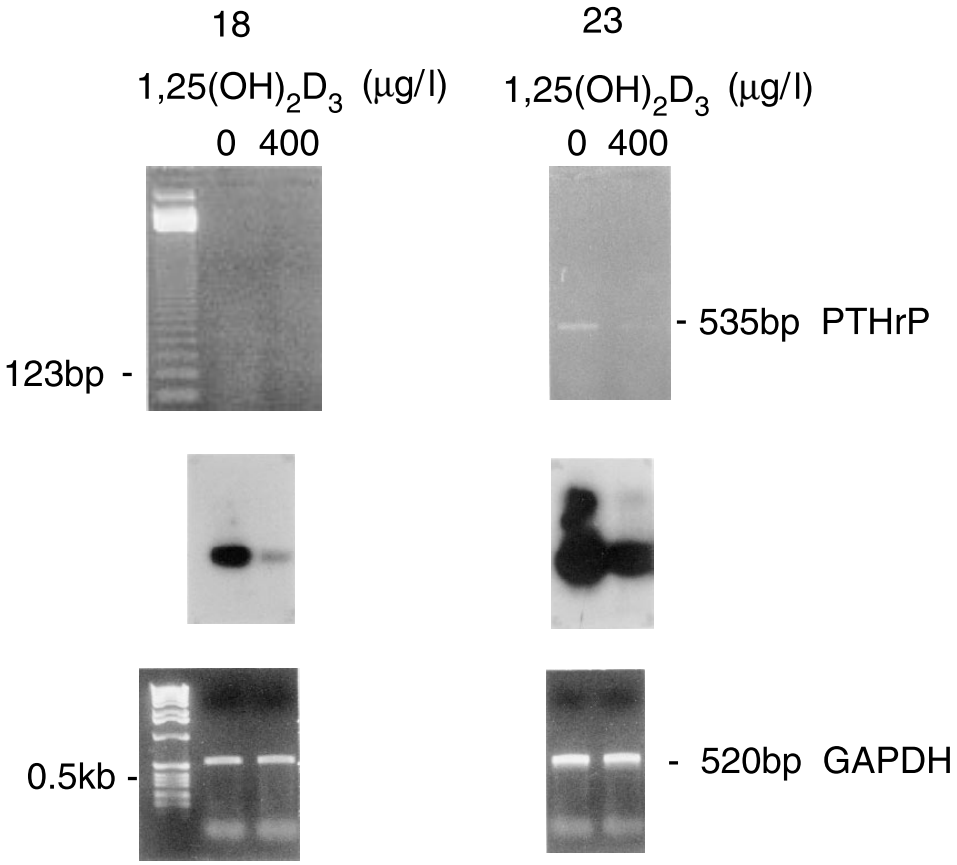

Fig. 4 (Top panels) RT-PCR amplification products obtained at 18 and 23 thermal cycles from cultured osteoblasts of MAS1 patient before (c) and after (+ D_3) treatment with 400 μg/l $1,25(OH)_2D_3$. (Middle panels) Southern blot analysis of the PCR products. (Bottom panels) GAPDH constitutive control for RT-PCR.

of foetal and adult tissues. The protein is thought to be a local regulator of bone turnover, having effects on the activity of both osteoclasts and osteoblasts. We report that osteoblasts in culture derived from three patients with MAS secrete PTHrP and express PTHrP mRNA at a significantly higher level than control subjects. This level of mRNA expression and PTHrP secretion in MAS patients is the highest we have detected throughout all of our studies of cultured human osteoblasts (Walsh *et al*., 1994, 1995). Furthermore, the secretion of PTHrP protein, and expression of PTHrP mRNA by human osteoblasts was down-regulated *in vitro* by $1,25(OH)_2D_3$.

This is the first evidence of abnormal secretion of PTHrP from osteoblasts derived from MAS patients. Whilst many other factors may be overexpressed in MAS bone our findings point to a contributory role for the peptide in the aetiology of the bone disease seen in such patients. Albright (1947) commented that the histological appearance of the bone in MAS was reminiscent of long-standing hyperparathyroidism and that one or more of the cases of osteitis fibrosa generalisata thought to be due to hyperparathyroidism were cases of MAS. No evidence of elevated PTH has been reported in MAS patients and our patients had normal plasma PTH (1–84) and PTHrP concentrations. PTHrP shares many of the activities of PTH including stimulation of adenylate cyclase activity in osteoblasts (Suva *et al*., 1987), stimulation of bone resorption *in vitro* (Evely *et al*., 1991) and *in vivo* (Horiuchi *et al*., 1987). These effects are mediated through the PTH receptor since PTH and PTHrP demonstrate sequence homology for eight of the first 13 amino acids at the amino terminal end of the protein (Suva *et al*., 1987). Therefore the histological picture may represent long-standing exposure to high concentrations of locally produced PTHrP acting in an autocrine, paracrine or intracrine fashion on bone cells. Specifically, N-terminal PTHrP fragments may play a role in bone marrow fibrosis whilst mid-molecule and C-terminal fragments, whose biological activity has not been fully documented, could contribute to other histological signs in MAS. Immunohistochemical staining (Fig. 5) provides confirmation of local and sporadic

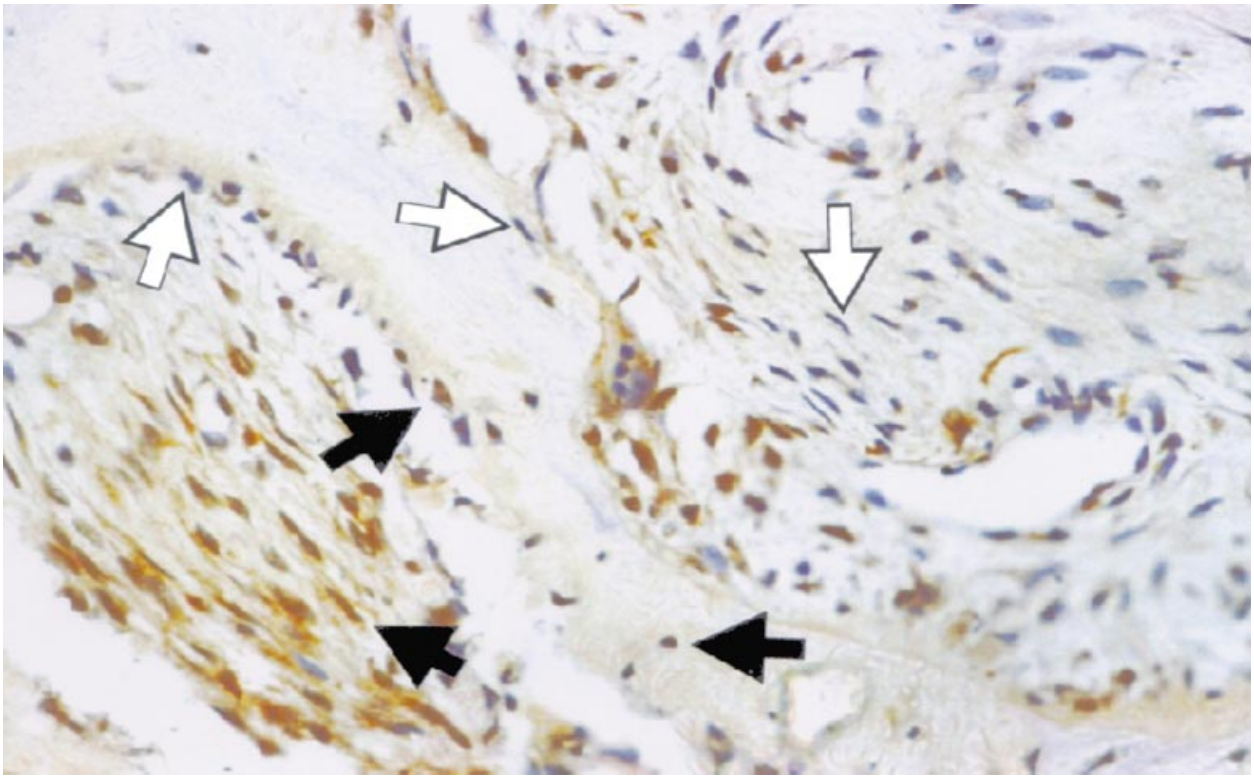

Fig. 5 Immunohistochemical localization (brown staining) of PTHrP(43–52) in bone from MAS3 prior to treatment with $1,25(OH)_2D_3$ Sporadic staining was observed in osteoblasts, osteocytes and marrow fibroblasts. Examples of cells staining positively are shown by black arrows, and negatively staining cells by white arrows.

production of PTHrP in the bone microenvironment in MAS. Some cells of the osteogenic lineage, including preosteoblasts, osteoblasts and osteocytes were found to stain strongly for PTHrP(43–52) in these sections.

Mutations in G_s have been identified in sporadic endocrine tumours (Vallar *et al*., 1987) and pituitary tumours (Spada *et al*., 1992) of MAS patients leading to constitutive activation of adenylate cyclase promoting autonomous function and clonal expansion of cells carrying the mutation (Landis *et al*., 1989). This mutation has previously been detected in osteoblast progenitor cells (Shenker *et al*., 1994), and in this study we have detected the presence of the mutations in genomic DNA from whole bone and some populations of osteoblasts. Our findings of low, and variable, expression of the mutation in cultured osteoblasts are entirely consistent with those of other authors (Yamamoto *et al*., 1996; Riminucci *et al*., 1997). Despite the apparently low abundance of mutant cells in the cultures of osteoblasts studied, we observed significantly elevated production and expression of PTHrP. These results suggest that overexpression of PTHrP is not confined to osteoblasts carrying the MAS mutation. One possible explanation for our findings is that the mutant cells, even in low abundance, influence the expression of PTHrP by wild-type osteoblasts. Other authors have reported increased production of interleukin 6 in cultures of MAS osteoblasts in which mutant cells were in a minority (Yamamoto *et al*., 1996). In addition, Bianco *et al*. (1998) have shown that mutant cells influence the behaviour of normal cells resulting in the production of abnormal bone with all of the histological features of fibrous dysplasia. The mechanism by which mutant cells mediate this abnormal bone formation may be a result of elevated production of local mediators including cytokines, or cAMP acting through gap junctions. Our data implicates PTHrP as a potential factor involved locally in the aetiology of bone lesions.

Hyperphosphaturic hypophosphataemic rickets or osteomalacia is commonly observed in patients with MAS (Lever & Pettingale, 1993) and is classically treated with cholecalciferol and inorganic phosphate but generally shows little response (Schwindinger & Levine, 1993). We have shown that $1,25(OH)_2D_3$ decreased steady state levels of PTHrP mRNA

Table 4 Biochemical measurements pre and post $1,25(OH)_2D_3$ treatment

	Time pre- or post-treatment (months)	Alk Phos (U/l)	Adj Ca (mmol/l)	PO_4 (mmol/l)	PTH (1–84) (pmol/l)	PTHrP (pmol/l)	25-hydroxy vitamin D_2 (μg/l)	25-hydroxy vitamin D_3 (μg/l)	$1,25(OH)_2D_3$ (ng/l)
Reference range	n/a	30–120	2·20–2·60	0·70–1·40	1·1–6·9	<0·7–2·6	<10	5–25	18–62
MAS 1	−5	1180	2·57	0·77	2·6	<0·7	3	81	16
	−1	1640	2·44	0·79	2·2	<0·7	3	40	15
	+3	960	2·38	0·88	2·1	<0·7	6	32	13
	+6	748	2·38	0·73	NA	NA	NA	NA	NA
	+10	832	2·36	0·66	1·5	<0·7	8	30	19
	+12	662	2·44	0·93	1·8	NA	10	20	25
	+16	448	2·47	0·94	NA	NA	12	15	28
MAS 2	−2	5820	2·45	0·63	3·2	<0·7	3	24	24
	+2	4181	2·42	0·60	3·3	<0·7	3	28	29
	+4	4020	2·43	0·55	3·5	<0·7	3	30	30
	+8	3947	2·44	0·61	2·7	NA	5	10	NA
	+12	3118	2·54	0·66	2·1	NA	3	12	32
MAS 3*		2381	2·54	0·94	1·2	NA	3	50	65
MAS 4	−1	2678	2·44	0·88	3·4	<0·7	14	20	28
	+2	1564	2·44	0·93	3·5	<0·7	12	15	30
	+4	1352	2·38	0·94	2·8	NA	12	18	33
	+9	1116	2·42	0·92	2·7	<0·7	16	10	31
	+12	1084	2·54	0·94	2·1	<0·7	10	20	40

NA, not analysed.
*MAS 3 was not treated with $1,25(OH)_2D_3$.

expression and inhibited PTHrP secretion *in vitro*. Subsequently we reduced the circulating concentration of alkaline phosphatase in MAS1, MAS2 and MAS4 by $1,25(OH)_2D_3$ treatment indicating an *in vivo* biochemical response to therapy. This decrease in alkaline phosphatase levels in response to commencement of $1,25(OH)_2D_3$ is not characteristic of the response of some patients with osteomalacia. However, our data may represent a specific response for MAS patients. $1,25(OH)_2D_3$ can control transcription of several vitamin D-dependent genes (Ikeda *et al*., 1989; Inoue *et al*., 1993) including the PTHrP gene which contains consensus regulatory motifs for $1,25(OH)_2D_3$. PTHrP gene transcription is down-regulated by $1,25(OH)_2D_3$ in a human C cell line (Ikeda *et al*., 1989) and human T cells (Ikeda *et al*., 1993). *In vivo*, long-term treatment should result in a significant change in the histological picture and improve the metabolic bone disease in MAS patients.

We have demonstrated a significant elevation of PTHrP mRNA expression and PTHrP secretion by osteoblasts derived from the bone of three McCune Albright syndrome patients. This has offered an explanation for some of the histological abnormalities observed in these patients. In addition, we have shown that treatment with $1,25(OH)_2D_3$ can alter osteoblast function *in vitro* and *in vivo*. These findings may provide a new approach to the therapy of McCune Albright syndrome.

Acknowledgements

This work was funded by SPARKS and by Action Research. We are grateful to Ms B. Wlodarski for histology.

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