Specific down-regulation of parathyroid hormone (PTH) receptors and responses to PTH by tumour necrosis factor α and retinoic acid in UMR 106-06 osteoblast-like osteosarcoma cells

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Parathyroid hormone (PTH) and PTH-related protein (PTHrP) act via PTH receptors in bone to stimulate bone resorption. Bone resorption is also stimulated by certain cytokines, which are produced in bone and bone marrow. The effects of such cytokines on the PTH-receptor system were studied in the osteoblast-like osteosarcoma cell line UMR 106-06. ^{125}I -labelled PTHrP-(1–84)-peptide bound specifically to the cells, and PTHrP-(1–34) and -(1–84) competed with equimolar affinity for binding to UMR 106-06 cells. The specific binding of ^{125}I -PTHrP-(1–84) could be completely blocked by PTH. Therefore ^{125}I -PTHrP-(1–84) bound to a classical PTH receptor in UMR 106-06 cells. Preincubation for 3 days with either tumour necrosis factor α (TNF α) or retinoic acid (RA) both decreased the specific binding of ^{125}I -PTHrP-(1–84) to about 40 % of control levels. These effects were specific for PTH binding, since there was little effect on ^{125}I -salmon-calcitonin binding. Both TNF α and RA required 24 h exposure to cells to produce a measurable effect. The decrease in ^{125}I -PTHrP-(1–84) binding was due to a reduced number of binding sites, with little apparent change in affinity. Half-maximal effects were seen with 1 ng of TNF α /ml, whereas 1 μM -RA was needed to observe the loss of PTH receptors. Combinations of RA and TNF α produced a greater effect than that of either agonist alone. The loss of PTH receptors was accompanied by a specific loss of PTH-stimulated cyclic AMP production. Preincubation with TNF α increased the basal plasminogen activator (PA) activity in the cells and decreased the amplitude of the response of PA activity to PTH compared with control cells. Furthermore TNF α decreased sensitivity to PTH (50 % stimulation of PA activity with 0.1 nM-PTH in control cells versus 50 % stimulation with 0.3 nM-PTH in TNF α -treated cells). In contrast, TNF α pretreatment increased the amplitude of the response of PA activity to calcitonin, whereas sensitivity to calcitonin was not altered. These data are consistent with a specific down-regulation of PTH receptors in osteoblast-like UMR 106-06 cells after exposure to TNF α or RA. The loss of PTH receptors is accompanied by a decreased responsiveness to PTH, as measured with the PA system in these cells. A loss of PTH receptors could modulate PTH responses in osteoblasts, either in the local control of bone formation and resorption, or in pathological conditions such as humoral hypercalcaemia of malignancy.

INTRODUCTION

Parathyroid hormone (PTH) acts directly upon osteoblasts, which then activate osteoclasts by a mechanism that is not yet understood. Certain cytokines produced in bone and bone marrow also stimulate bone resorption [1,2] by acting on osteoblasts [3,4]. The question arises of whether locally produced cytokines might regulate PTH receptor levels or PTH receptor function in the osteoblast. Such an interaction might contribute to the local regulation of bone formation and resorption, by which bone adapts locally to different forces and stresses. Indeed, preincubation with transforming growth factor α (TGF α) or epidermal growth factor has been shown to decrease subsequent cyclic AMP responses to PTH by up to 50 % in osteoblast-like osteosarcoma cells [5].

Another factor known to stimulate bone resorption and inhibit bone formation is tumour necrosis factor α (TNF α) [1], a cytokine that is produced by cells of the monocyte/macrophage lineage [6], by mononuclear phagocytes [7] and also by activated osteoblasts [8]. The closely related cytokine lymphotoxin (TNF β), which interacts with the same receptors, has similar effects on bone resorption [9]. TNF α stimulates DNA synthesis and inhibits collagen synthesis in osteoblasts [10–12]. In contrast to these

effects, which reflect inhibition of expression of differentiated osteoblastic features, TNF α potentiates retinoic acid (RA)-induced alkaline phosphatase activity in a preosteoblastic cell line [13]. RA is thought to have an important function as a morphogen in the development of the limb bud [14,15]. *In vitro* it inhibits the growth of osteoblast-like osteosarcoma cells [16,17], induces vitamin D₃ receptors [18] and has differential effects on alkaline phosphatase activity in bone-derived cells. In two similar preosteoblastic cell lines it induces alkaline phosphatase activity, while in cells with high constitutive levels of alkaline phosphatase a decrease is seen after RA treatment [19,20].

The present study examined the effects of various factors, in particular RA and TNF α , on the PTH receptor status in the osteoblastic osteosarcoma cell line UMR 106-06. We chose the subclone UMR 106-06 because, in addition to PTH receptors, these cells possess calcitonin (CT) receptors, which serve as a control in studies of specific regulation of the PTH receptor. In addition, we examined the specific regulation by TNF α of PTH-responsive adenylate cyclase activity and CT- and PTH-stimulated plasminogen activator (PA) activity. We demonstrate here that RA and TNF α specifically down-regulate PTH receptors, PTH-responsive cyclic AMP production and PTH-dependent PA activity in UMR 106-06 cells.

Abbreviations used: PTH, parathyroid hormone; PTHrP, PTH-related protein; TGF α , transforming growth factor α ; TNF α , tumour necrosis factor α ; RA, retinoic acid; (s)CT, (salmon) calcitonin; PA, plasminogen activator; MEM, minimal essential medium; IBMX, isobutylmethylxanthine.

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MATERIALS AND METHODS

Materials

Recombinant PTH-related protein (PTHrP)-(1-84) was expressed and purified as described [21]. RA (all-*trans*) was purchased from Sigma (St. Louis, MO, U.S.A.). Recombinant TNF α , interleukin 1 α and TGF α were generously provided by Genentech Inc. (South San Francisco, CA, U.S.A.). PTHrP-(1-34) was synthesized as described [22]. Human PTH-(1-34) was bought from Auspep (Melbourne, Australia). Recombinant human PTH-(1-84) was kindly given by Professor K. Gautvik (University of Oslo, Norway). Minimal essential medium (MEM) was obtained from Flow Laboratories (ICN Biomedicals Australasia, Sydney, Australia), fetal calf serum was from CSL (Melbourne, Australia) and BSA was from Armour Pharmaceutical Company (Rorer, Sydney, Australia). Isobutylmethylxanthine (IBMX) was obtained from Sigma.

Cell culture

UMR 106-06 cells were maintained in culture as described previously [23]. For binding experiments, cells were subcultured into 12-well plates (Costar, Cambridge, MA, U.S.A.) at concentrations of $(1-3) \times 10^5$ cells/well. Cells between passages 15 and 40 were used for the experiments. Treatment was started after the cells had attached to the wells overnight and agonists were added in 1% of the total volume. Controls for RA treatment contained concentrations of ethanol up to 0.1%, which did not affect the binding characteristics of the cells.

Iodination of PTHrP-(1-84)

Purified PTHrP-(1-84) (3 μ g) was iodinated using 1 mCi of 125 I and 10 μ g of chloramine T for 30 s. Specific radioactivities of 250–300 μ Ci/ μ g were routinely achieved. The reaction was terminated by the addition of cysteine hydrochloride (Ajax, Sydney, Australia). After separation of iodinated hormone from free iodine on SepPak C $_{18}$ cartridges (Waters, Milford, MA, U.S.A.), further purification was achieved by reverse-phase h.p.l.c. An RP 300 Aquapore C $_8$ guard column (Activon, Thornleigh, Australia) and an acetonitrile gradient of 0–50% (v/v) over 50 min was used. The peak fractions were stored at -20°C for up to 4 weeks without loss of activity. The iodinated hormone was run on SDS/15% PAGE as described by Laemmli [24] for size determination. Gel electrophoresis and autoradiography of the gel were performed as described previously [25].

Binding studies

For binding studies, culture media were removed from the cell monolayers and replaced with 900 μ l of MEM containing 0.1% BSA. After prewarming at 37°C for 15 min, 125 I-PTHrP-(1-84) and different concentrations of unlabelled polypeptides were added in 50 μ l volumes. Non-specific binding was determined by incubating with 125 I-PTHrP-(1-84) in the presence of 1 μ M unlabelled PTHrP-(1-84). Incubation was continued at 37°C for 60 min. The supernatants were then removed and the cells were washed three times with cold phosphate-buffered saline (140 mM-NaCl/3 mM-KCl/8 mM-Na $_2$ HPO $_4$ /1.5 mM-KH $_2$ PO $_4$). Cell-bound 125 I-PTHrP-(1-84) was determined by solubilizing cells in 1 ml of 0.5 M-NaOH and measurement of radioactivity in a γ -radiation counter (Cobra Autogamma 5005; Packard, Downer's Grove, IL, U.S.A.) with an efficiency of 80%. Competition isotherms were analysed using the LIGAND program [26]. Values are expressed as means $\pm$ S.E.M. of triplicate determinations.

Determination of cyclic AMP

UMR 106-06 cells were grown to confluency in 12-well plates. After removal of media the cells were preincubated with MEM containing 0.1% BSA and 1 mM-IBMX for 20 min at 37°C . Cells were then incubated with hormones in the same medium for 10 min. After removal of media, cyclic AMP was extracted with ethanol, which was transferred to tubes, dried down and reconstituted in assay buffer. Cyclic AMP was determined by r.i.a. as described previously [27].

PA assay

PA activity was measured essentially as described elsewhere [28,29]. UMR 106-06 cells were subcultured at 4×10^4 cells/0.2 ml on to 125 I-fibrin (Amersham International, Sydney, Australia)-coated wells of 96-well tissue culture plates (Linbro, McLean, VA, U.S.A.) prepared and activated as described [28]. After the cells had become attached over 24 h, the medium was changed to MEM/0.1% BSA for 6 h. Then media were removed and the cells were stimulated with different amounts of hormone in MEM/0.1% BSA. Fibrinolytic activity was measured in the presence and the absence of 1.5 μ g of plasminogen/ml (Sigma) to allow the generation of plasmin, and plasminogen-dependent fibrinolytic activity was determined. After 36 h an aliquot was removed and counted for radioactivity.

Statistics

Values are means $\pm$ S.E.M. of triplicates determinations. Where indicated, statistical evaluation was performed using Student's *t* test; $P < 0.05$ was considered significant.

RESULTS

Characterization of PTH receptors on UMR 106-06 cells using 125 I-PTHrP-(1-84)

Iodination of PTHrP-(1-84) with 125 I using the chloramine T method resulted in 70–90% incorporation of 125 I into protein, as determined by precipitation with 10% trichloroacetic acid. The iodinated molecule eluted as a single peak of radioactivity on h.p.l.c. The elution time of iodinated PTHrP-(1-84) was 3 min longer than that of the uniodinated PTHrP-(1-84) species, and allowed the purification of carrier-free 125 I-PTHrP-(1-84) (Fig. 1). Autoradiography following PAGE revealed a single radioactive band which co-migrated with unlabelled PTHrP-(1-84) (results not shown), and confirmed that the iodination procedure did not degrade the molecule.

Fig. 1. Reverse-phase h.p.l.c. purification of 125 I-labelled PTHrP-(1-84)

125 I-labelled PTHrP-(1-84) was purified on h.p.l.c. using an RP-300 guard column and a flow rate of 1 ml/min. Aliquots of 5 μ l from 1 min fractions were counted for radioactivity in a γ -radiation counter for 0.1 min. The arrow indicates the elution position of unlabelled PTHrP-(1-84).

Fig. 2. Competition of ^{125}I -PTHrP-(1-84) with PTHrP-(1-34) and PTHrP-(1-84) for binding to UMR 106-06 cells

Incubation was for 1 h at 37 °C as described, using 150 000 c.p.m. of ^{125}I -PTHrP-(1-84)/ml (0.25 ng/ml). PTHrP-(1-34) ($\square$) and PTHrP-(1-84) ($\blacksquare$) competed equally for binding. Maximal binding was 5% of the added counts, and non-specific binding was approx. 10% of the total binding. Values are means $\pm$ S.E.M. of triplicate determinations. These results are representative of four independent experiments.

Fig. 3. Time course of down-regulation of ^{125}I -PTHrP-(1-84) binding after preincubation with $\text{TNF}\alpha$ or RA

UMR 106-06 cells were preincubated for different time intervals with 1 μM -RA ($\blacksquare$) or 10 ng of $\text{TNF}\alpha$ /ml ($\square$). Binding in the presence of 300 000 c.p.m. of ^{125}I -PTHrP-(1-84) (0.45 ng/ml) was measured as described and is expressed as a percentage of control binding, which was determined at each time point. Non-specific binding was 20% of total binding in control cells. Values are means $\pm$ S.E.M. and represent two independent experiments.

Binding to UMR106-06 cells

^{125}I -labelled PTHrP-(1-84) bound specifically to UMR 106-06 cells. Maximal binding was reached after 60 min at 37 °C, and

Fig. 4. Effects of preincubation with $\text{TNF}\alpha$ or RA on PTH receptor number and affinity

UMR 106-06 cells were preincubated with $\text{TNF}\alpha$ (10 ng/ml) or RA (1 μM) for 48 h before ^{125}I -PTHrP-(1-84) binding was determined as described using 300 000 c.p.m. of ^{125}I -PTHrP-(1-84)/ml (0.45 ng/ml). (a) ^{125}I -PTHrP-(1-84) binding and competition with unlabelled PTHrP-(1-84) in control cells ($\bullet$), $\text{TNF}\alpha$ -treated cells ($\square$) and RA-treated cells ($\blacksquare$). (b) Data after transformation with the program LIGAND [26]. Binding affinity (K_D) was 8.2×10^{-9} M for control cells, 4.8×10^{-9} M for $\text{TNF}\alpha$ -treated cells and 7.3×10^{-9} M for RA-treated cells. Receptor numbers were 75 000/cell for controls, 30 100/cell for $\text{TNF}\alpha$ -treated cells and 28 100/cell for RA-treated cells. These results were similar in two independent experiments.

cells bound approx. 5% of the added label. Synthetic PTHrP-(1-34) and recombinant PTHrP-(1-84) were equipotent in competing for binding to the cells (Fig. 2), confirming that the first 34 amino acids of the protein are sufficient for receptor binding. Human PTH-(1-34) and bovine PTH-(1-84) were also able to completely displace specific binding to the UMR106-06 cells at 1 μM , while unrelated peptides, including sCT, human calcitonin-gene-related peptide, atrial natriuretic factor-(1-28) and angiotensin II did not show any competition for ^{125}I -PTHrP-(1-84) binding at these concentrations (results not shown). These results are consistent with the binding of ^{125}I -PTHrP-(1-84) to a classical PTH receptor on UMR 106-06 cells.

Fig. 5. Effect of a 48 h preincubation with different concentrations of TNF α on ^{125}I -PTHrP-(1-84) binding in UMR 106-06 cells

Cells were treated for 48 h with TNF α at the concentrations indicated and (a) binding of ^{125}I -PTHrP-(1-84) using 240 000 c.p.m. of labelled ligand/ml (0.36 ng/ml) and (b) cell number were measured. Points are means $\pm$ S.E.M. of triplicate determinations. Non-specific binding was 25% of total binding in control cells.

Effects of TNF α and RA upon binding of ^{125}I -PTHrP-(1-84)

In order to determine whether agents that stimulate bone resorption could regulate the PTH receptor, UMR 106-06 cells were preincubated for 3 days with several cytokines and other bone-activating agents. Preincubation with RA or TNF α consistently resulted in a marked decrease in ^{125}I -PTHrP-(1-84) binding, to approx. one-third of specific binding. To determine the specificity of these effects upon the PTH receptor, binding of ^{125}I -sCT was measured. The binding of ^{125}I -sCT to the cells was not altered by TNF α and was only slightly decreased by preincubation with RA. Preincubation with neither TGF α (10 ng/ml) nor interleukin 1 (10 ng/ml) led to marked changes in ^{125}I -PTHrP-(1-84) or ^{125}I -sCT binding. TNF α did not consistently change the growth rate of the cells in this experiment, whereas RA inhibited growth as previously described [17]. The time course of the effects of these factors on the PTH binding capacity of the cells is shown in Fig. 3; both RA and TNF α required 24 h to exert detectable effects. In the continued presence of RA or TNF α , the PTH-binding capacity continued to decline for a further 2 days, by which time it was approx. one-third of the specific binding of control cells. The decrease in ^{125}I -PTHrP-(1-84) binding induced by either RA or TNF α was primarily due to a decreased average receptor number per cell. Competition

Table 1. Effects of combinations of TNF α and RA on ^{125}I -PTHrP-(1-84) binding to UMR 106-06 cells

Cells were incubated for 48 h before binding was measured as described using 270 000 c.p.m. of ^{125}I -PTHrP-(1-84) (0.4 ng/ml). Total and non-specific binding, and cell number, were measured for every treatment. Non-specific binding was 18% of total binding to control cells. Values are means $\pm$ S.E.M. of triplicates and represent specific binding. Binding after treatment with a combination of 10 ng of TNF α /ml and 1 μM -RA was significantly different from binding after either treatment alone (* $P < 0.0005$). Binding after treatment with 0.1 ng of TNF α /ml or 0.1 μM -RA alone was not significantly different from the control; however, a combination of 0.1 ng of TNF α /ml and 0.1 μM -RA resulted in significantly decreased binding compared with the control ($\dagger P < 0.0005$).

Cell treatment	^{125}I -PTHrP-(1-84) bound (fmol/ 10^6 cells)
Control	16.1 $\pm$ 0.2
TNF α (10 ng/ml)	5.6 $\pm$ 0.4
RA (1 μM)	5.6 $\pm$ 0.1
TNF α (10 ng/ml) + RA (1 μM)	2.9 $\pm$ 0.0*
TNF α (0.1 ng/ml)	15.8 $\pm$ 0.1
RA (0.1 μM)	16.2 $\pm$ 1.5
TNF α (0.1 ng/ml) + RA (0.1 μM)	12.8 $\pm$ 0.3†

Fig. 6. Effect of TNF α or RA pretreatment on the cyclic AMP response to PTH in UMR 106-06 cells

Cells were pretreated with 10 ng of TNF α /ml (■) or 1 μM -RA (○) for 48 h; control cells were incubated in medium alone (□). Then media were changed to MEM containing 0.1% BSA and 1 mM-IBMX for 20 min. Different concentrations of PTH were added for 10 min and cyclic AMP was extracted as described. Cyclic AMP was measured with a r.i.a. [27].

curves with unlabelled PTHrP-(1-84) before and after treatment for 48 h with RA or TNF α (Fig. 4a) were transformed using the program LIGAND [26]. These plots showed straight lines, an indication for one non-interacting class of binding sites (Fig. 4b). The receptor concentration in control cells was 75074 ± 4175 per cell, and preincubation with TNF α or RA resulted in a marked down-regulation of the number of binding sites (30100 ± 1866 per cell after 10 ng of TNF α /ml for 48 h; 28100 ± 2672 per cell after 1 μM -RA for 48 h), with little apparent change in affinity (K_d 8.2×10^{-9} M for control cells; K_d 4.8×10^{-9} M for TNF α -treated cells and K_d 7.3×10^{-9} M for RA-treated cells) (Fig. 4b). The effects of TNF α were dose-dependent, with half-maximal down-regulation of PTH binding seen at 1 ng of TNF α /ml (Fig.

Fig. 7. Effect of pretreatment with $\text{TNF}\alpha$ on PTH-stimulated PA activity

UMR 106-06 cells were pretreated with 10 ng of $\text{TNF}\alpha/\text{ml}$ for 48 h, before stimulation with different concentrations of PTH. (a) After 36 h, the plasmin-dependent solubilization of ^{125}I -fibrin was measured in control cells ($\circ$) and $\text{TNF}\alpha$ -treated cells ($\bullet$). (b) PTH-stimulated release of radioactivity expressed as a percentage of the maximally released radioactivity. The experiment shown is representative of three independent experiments.

Fig. 8. Effect of pretreatment with $\text{TNF}\alpha$ on sCT-stimulated PA activity

UMR 106-06 cells were pretreated with 10 ng of $\text{TNF}\alpha/\text{ml}$ for 48 h, before stimulation with different concentrations of sCT. (a) After 36 h, the plasmin-dependent solubilization of ^{125}I -fibrin was measured in control cells ($\circ$) and $\text{TNF}\alpha$ -treated cells ($\bullet$). (b) sCT-stimulated release of radioactivity expressed as a percentage of the maximally released radioactivity. The experiment shown is representative of three independent experiments.

5a). An accompanying small increase in cell number was maximal (about 10%) with 10 ng of $\text{TNF}\alpha/\text{ml}$ (Fig. 5b). In contrast, a RA concentration of $1 \mu\text{M}$ was required for maximal down-regulation of PTH binding, and concentrations below this had little consistent effect (results not shown). A combination of RA and $\text{TNF}\alpha$ showed an additive effect, as maximal concentrations of the agents together had a greater effect than each of them alone. Furthermore, concentrations of the two agents which did not produce any down-regulation of PTH binding by themselves yielded a significant effect when they were administered together (Table 1).

Effects of $\text{TNF}\alpha$ and RA upon cellular responses to PTHrP and CT

Preincubation of UMR 106-06 cells with RA or $\text{TNF}\alpha$ also resulted in a marked decrease in the cyclic AMP response to PTH (Fig. 6). In contrast, the cyclic AMP response to sCT was not altered after $\text{TNF}\alpha$ or RA treatment (results not shown). These results are consistent with the loss of PTH receptors on treatment with these agents.

Further experiments were performed to study the biological significance of the loss of PTH binding. Both PTH and CT are known to stimulate PA activity in UMR 106-06 cells through a cyclic AMP-dependent mechanism [29]. As shown in Fig. 7(a), PTH stimulated plasminogen-dependent fibrinolysis in a dose-

dependent manner in UMR 106-06 cells. Pretreatment for 48 h with $\text{TNF}\alpha$ (10 ng/ml) led to increased PA activity (Fig. 7a). However, the PA response to PTH was decreased compared with that in control cells (stimulation by PTH of 36000 c.p.m. after $\text{TNF}\alpha$ treatment versus 48000 c.p.m. in control cells). If the PTH-responsive PA activity was expressed as a percentage of the maximal response to PTH in $\text{TNF}\alpha$ -treated and control cells, a decrease in the sensitivity to PTH after $\text{TNF}\alpha$ treatment was observed (Fig. 7b; 50% stimulation with 0.1 nM-PTH in control cells; 50% stimulation with 0.3 nM-PTH in $\text{TNF}\alpha$ -treated cells), a change which correlated well with the degree of loss of PTH receptors.

In contrast, $\text{TNF}\alpha$ pretreatment enhanced the CT-responsive PA activity (stimulation by sCT of 44000 c.p.m. in $\text{TNF}\alpha$ -treated versus 24000 c.p.m. in control cells) (Fig. 8a). Expression of the CT-responsive PA activity as a percentage of the maximal CT response showed no change in sensitivity to CT after $\text{TNF}\alpha$ treatment (Fig. 8b; 50% stimulation with 0.02 nM-sCT in both control and $\text{TNF}\alpha$ -treated cells), again consistent with the unchanged CT receptor status.

RA treatment of the cells led to maximally increased PA activity, which could not be further enhanced with PTH treatment (results not shown), so that the effects of RA on the response to PTH could not be studied.

DISCUSSION

Several studies using C-terminally tyrosinated synthetic peptides of up to 40 amino acids have shown that the *N*-terminal part of PTHrP binds to classical PTH receptors in kidney membranes and osteosarcoma cells [30–33]. Longer recombinant PTHrP molecules have become available [21], and PTHrP-(1–84), iodinated using the conventional chloramine T method, binds specifically to rat osteoblasts in primary cultures as shown by autoradiography [34]. In the present work specific binding of ^{125}I -PTHrP-(1–84) to UMR 106-06 cells was inhibited by equimolar amounts of PTHrP-(1–34) and PTHrP-(1–84), thus indicating that the specific binding portion of this molecule is contained within the first 34 residues. The longer molecule PTHrP-(1–84) was chosen for these PTH receptor studies as it consistently showed lower non-specific binding compared with ^{125}I -labelled PTHrP-(1–34) (results not shown). The affinity of the PTH receptor in UMR 106-06 cells (K_d 8×10^{-9} M) is similar to that found in two previous studies which used the UMR 106 strain and either ^{125}I -labelled [Tyr³⁶]PTHrP-(1–36) [31] or ^{125}I -labelled rat PTH-(1–34) [35] as labelled ligand.

UMR 106-06 cells are a subclone of the UMR 106 cell line, a rat osteosarcoma cell line with osteoblastic features including high alkaline phosphatase activity [36], vitamin D receptors [37] and collagenase production [38]. Late passages of these cells were found to be responsive to both PTH and CT, and subcloning resulted in a clonal cell line which is responsive to both CT and PTH [23]. It is unclear why these cells express CT receptors, as the osteoblast is not normally considered to be the target cell for CT in bone. In order to examine a specific effect on the PTH receptor system, however, these cells are useful because the CT receptor status can be used as a control.

TNF α and RA were both found to down-regulate specifically PTH binding in this cell line (Fig. 3). This was primarily due to a change in receptor number, as affinity was not altered. The time courses of action of TNF α and RA suggest that these agents act at the genomic level to decrease transcription or by decreasing PTH receptor mRNA stability. The necessary mechanistic studies to test these possibilities can be carried out when the PTH receptor is cloned. The effects of the two factors seem to be additive, as concentrations which did not show any effect by themselves were able to down-regulate PTH receptors in combination. The cyclic AMP response to PTH was diminished after treatment with either TNF α or RA (Fig. 6). This is in agreement with the observed decrease in receptor numbers.

In a previous report from this laboratory [39], treatment with 10 nM-RA for 4 days moderately increased the amplitude of adenylate cyclase responsiveness to PTH and changed the pattern of activation of protein kinase A isoenzymes. The most likely explanation for the decreased adenylate cyclase responsiveness to PTH seen after RA administration in our experiments is the 100-fold higher RA concentrations used. Importantly, 1 μM -RA was previously shown to be non-toxic to cells of the UMR 106 lineage [40]. Pretreatment with 10 nM-RA did not change the level of ^{125}I -PTHrP-(1–84) binding; therefore changes in G-protein levels or levels of the catalytic subunit of adenylate cyclase could account for the observed effect. In agreement with our observations, pretreatment with 1 μM -RA decreased PTH-responsive adenylate cyclase in ROS 17/2A cells; however, the magnitude of the effect was dependent on the serum addition in the culture [41]. A decrease in the PTH-stimulated adenylate cyclase response after TNF α treatment in UMR 106 cells has been reported [42] and this is consistent with the loss of PTH receptors described here. Pretreatment with interleukin 1, however, which was also reported to lower PTH-stimulated cyclic AMP production in that report, failed to produce an effect on

PTH receptors in UMR 106-06 cells. Similarly, treatment with TGF α , which decreased the cyclic AMP response to PTH in UMR 106 cells [5], did not significantly change PTH receptor binding in our experiments. Therefore regulation at the G-protein or the adenylate cyclase level is likely to account for the observed differences. In agreement with this hypothesis, TGF α decreased not only PTH-mediated but also isoprenaline- and forskolin-stimulated cyclic AMP production [5].

PTH- and sCT-stimulated PA activity was measured in UMR 106-06 cells to examine whether the specific down-regulation of PTH receptors was accompanied by a specific decrease in PTH-stimulated PA activity. This biological response seemed ideally suited, as both CT and PTH increase PA activity in these cells by a cyclic AMP-dependent mechanism [29]. RA increased PA activity to maximal levels, so that a further effect of PTH could not be observed. Preincubation with TNF α for 2 days diminished the PA response to PTH, while basal PA production was increased compared with control cells. The observed decrease in the sensitivity to PTH is consistent with the reduced number of PTH receptors. In contrast, the amplitude of the PA response to CT was increased after TNF α treatment, while the sensitivity of the response to CT was not altered, again consistent with the lack of effect of TNF α on CT receptor levels. The increased amplitude of the PA response to CT after TNF α treatment might be caused by the combined stimulation of PA activity by different intracellular pathways.

TNF α has previously been shown to increase bone resorption and inhibit bone formation [1,10]. The finding that it decreases PTH receptor number and sensitivity to PTH in osteoblast-like osteosarcoma cells can contribute to the understanding of the regulation of local bone turnover. PTH not only stimulates bone resorption but also increases bone formation when given in intermittent low doses [43,44]. Decreased sensitivity to PTH might affect bone formation more than it would affect bone resorption, as resorption is stimulated by both PTH and TNF α . How combinations of PTH and TNF α together act on bone resorption has not yet been studied, although synergistic stimulation of bone resorption has been shown for both PTH and TNF α with interleukin 1 [45,46].

TNF α - or RA-mediated PTH receptor regulation might contribute to the local processes of bone resorption and formation, which allow bone to adapt to varying stimuli. By decreasing osteoblast sensitivity to PTH, TNF α might promote an uncoupling of bone formation and resorption. Such uncoupling is a feature of humoral hypercalcaemia of malignancy [47], in which locally active cytokines are produced [48]; however, a role for TNF α in that syndrome has yet to be established.

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