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TUMOR NECROSIS FACTOR α INHIBITS THE STIMULATORY EFFECT OF THE PARATHYROID HORMONE-RELATED PROTEIN ON CYCLIC AMP FORMATION IN OSTEOBLAST-LIKE CELLS VIA PROTEIN KINASE C+

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Tumor necrosis factor α (TNF α) and parathyroid hormone-related protein (PTHrP) are both factors that have been implicated in the mechanism of hypercalcemia of malignancy. In this study we investigated the effect of TNF α on the PTHrP-stimulated accumulation of intracellular cyclic AMP in osteoblast-like cells. In the clonal cell line Saos-2 and in primary cell cultures from fetal rat calvaria, PTHrP-stimulated accumulation of cAMP was time- and dose-dependently inhibited by exposure to TNF α. Significant inhibition occurred at concentrations as low as 2 x 10^{-12} M and was maximal at 1 x 10^{-9} M. Inhibition was observed after 6 h and was maximal after 18 h. Inhibition by TNF α was probably mediated by protein kinase C , since the phorbol ester PMA mimicked the effect of TNF α, and the protein kinase C inhibitor H-7 completely abolished the effect of TNF α. In conclusion, these observations suggest a possible mechanism by which TNF α may modulate the effect of PTHrP on osteoblast function in the syndrome of humoral hypercalcemia of malignancy.

Hypercalcemia is a frequent complication of malignancy. It is now believed that parathyroid hormone-related protein (PTHrP) plays a major pathogenetic role in the syndrome of humoral hypercalcemia of malignancy (1,2). However, in many cases hypercalcemia does not correlate well with plasma levels of PTHrP (3). This indicates that additional factors might interfere with the hypercalcemic effects of PTHrP in these patients. One putative candidate for modulating the effects of PTHrP on bone metabolism is TNF α. This cytokine was initially described as a 17,000 MW protein with a variety of biological effects and a prominent role in tumor cachexia and inflammation (reviewed by: (4)).

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Abbreviations: cAMP, cyclic adenosinmonophosphate; H-7, 1-(5-isoquinoline-sulfonyl)-2-methylpiperazine dihydrochloride; PMA, Phorbol-12-myristate-13-acetate; PK-C, protein kinase C; PTHrP, parathyroid hormone-related protein; TNF α, tumor necrosis factor α.

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Very recently, it has been shown that TNF α plays in fact a pathogenetic role in hypercalcemia of malignancy in the rat Leydig cell tumor (5) and in a human squamous cell carcinoma transplanted in nude mice (6). These authors found that TNF α was not produced directly by the tumor but released from host immune cells in response to the tumor.

In the skeletal system, TNF α induces osteoclast formation (7), increases bone resorption in vitro (8), and causes hypercalcemia in mice when injected subcutaneously (9). TNF α also inhibits collagen synthesis in osteoblast-like cells (10,11). Moreover, osteoblast-like cells appear to be responsible for many of the effects of TNF α and PTHrP on bone resorption since these factors stimulate isolated osteoclasts to resorb bone only in the presence of osteoblast-like cells (12,13).

Stimulation of adenylate cyclase has long been the hallmark of the effect of PTH on the osteoblast metabolism (14). Similar increases in cAMP have also been observed in osteoblast-like cells upon stimulation with PTHrP (15). In previous studies it has been shown that other osteotropic factors such as epidermal growth factor, transforming growth factor α, and transforming growth factor β were able to decrease (16) or increase (17) PTH-stimulated adenylate cyclase activity in the clonal osteoblast-like cell line UMR 106. In this study we investigated the effect of TNF α on PTHrP-stimulated adenylate cyclase in osteoblast-like cells and tried to identify its mechanism of action .

MATERIALS AND METHODS

The osteosarcoma cell line Saos-2 (American Tissue Culture Collection, Rockville, Maryland) was maintained in monolayers with McCoy´s medium (Biochrom, Berlin, Germany) supplemented with 10 % fetal calf serum (Gibco, Eggenstein, Germany), 1 % L-Glutamin (200 mM), 1 % Hepes and antibiotics in a humified atmosphere of 95 % air / 5 % CO_2 at 37 °C. The cells were subcultured weekly using 0.05 % Trypsin and 0.02 % EDTA in Ca^{++} and Mg^{++} free PBS. For the experiments, cells were plated in 12-well tissue culture plates at 2.4 - 3 x 10^5 cells per well.

Primary cultures from rat calvaria were obtained from 21-day-old fetal rats (Thomae AG, Biberach, Germany). 5 sequential collagenase digestions were performed as described recently (18). Cells from fractions 3 - 5 were pooled and maintained in Dulbecco´s Modified Eagle´s Medium (DMEM, Gibco), supplemented with 15 % fetal calf serum , 1 % L-Glutamin , 1 % Hepes and antibiotics under same conditions as above. For the experiments, cells were plated in 24-well tissue culture plates at 0.6 - 1 x 10^5 cells per well and used in their first or second passage.

Stock solutions were prepared from recombinant human TNF α (generous gift from Dr. Kempeni, Knoll AG, Ludwigshafen, Germany), Phorbol-12-myristate-13-acetate (PMA; Sigma, Deisenhofen, Germany), pertussis toxin (Sigma), 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride (H-7; Sigma), and human PTHrP(1-86) (Bissendorf Biochemicals, Hannover, Germany).

For the stimulation of the adenylate cyclase, stock solutions were further diluted with assay buffer consisting of McCoy´s medium (Saos-2 cells) or DMEM (rat calvaria cells), each supplemented with 5 % Hepes, 1 % BSA, and 1 mM isobutyl-methylxanthine. Cells in monolayers were incubated for 15 min. After removal of the supernatant, accumulated intracellular cAMP was extracted with 1 ml of 95 % ethanol, pH 3. After 2 h, the alcohol was removed by evaporation and cAMP measured using a competitive protein binding assay (19).

All measurements were performed in triplicate wells. Data are expressed as mean ± SD. All experiments were performed independently at least 3 times.

RESULTS

Pretreating Saos-2 cells with TNF α for 24 h inhibited the PTHrP(1-86)-stimulated accumulation of intracellular cAMP (Fig. 1). The inhibitory effect was dose-dependent and occurred at concentrations as low as 2 x 10^{-12} M TNF α. The inhibition was maximal at 1 x 10^{-9} M TNF α. PTHrP (1-34) and hPTH (1-34) were 2- to 4-fold more potent than PTHrP (1-86), but TNF α inhibited the accumulation of cAMP of all 3 peptides identically. The cell numbers and the basal cAMP accumulation did not change in the presence of TNF α (6.56 ± 0.25 x 10^5 cells per well without TNF α vs. 6.58 ± 0.26 x 10^5 cells per well in the presence of 10^{-8} M TNF α; basal intracellular cAMP content was 10.9 ± 1.2 pmol/1 Mio cells vs. 9.6 ± 0.6 pmol/1 Mio cells). The inhibitory effect of TNF α on cAMP accumulation stimulated with different doses of PTHrP was also observed in primary cultures of osteoblast-like cells from fetal rat calvaria (Fig. 2).

Inhibition of PTHrP-stimulated accumulation of cAMP in Saos-2 cells by TNF α was first observed after 12 h and was maximal after 18 h (Fig. 3, left panel). Under conditions of constant exposure of the cells to TNF α a maximal inhibition was still observed after 48 h. Upon removal of TNF α inhibition disappeared in a linear manner and was almost gone after 30 h (Fig. 3, right panel).

In many cells the inhibition of adenylate cyclase is mediated by a guanine nucleotide-binding protein (G_i) that can be inactivated by pertussis toxin. Coincubating Saos-2 cells with pertussis toxin increased the PTHrP-stimulated accumulation of cAMP up to 54% but had no effect on the inhibitory potency of TNF α at any concentration (Table 1).

The effect of TNF α on accumulation of cAMP in PTHrP-stimulated Saos-2 cells could be mimicked by incubating the cells for an identical period of time (24 h) with the phorbol ester PMA, an activator of PK-C (Fig. 4). Since this suggested a possible involvement of PK-C, we

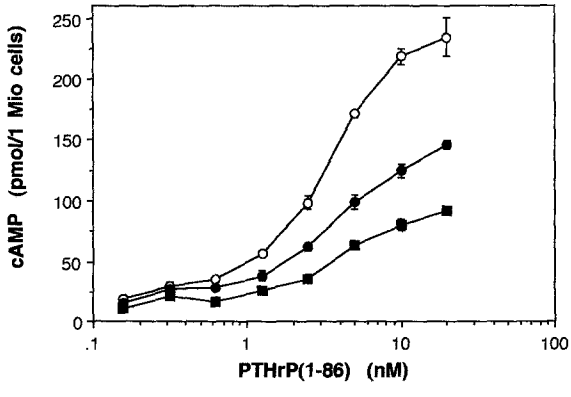

Figure 1. Stimulation of the accumulation of cAMP in Saos-2 cells by PTHrP with and without TNF α.
The cells were pretreated with vehicle alone (○), 5 x 10^{-11} M TNF α (●), or 10^{-9} M TNF α (■) for 24 h. The *y*-axis shows the accumulation of cAMP during a 15 min exposure of the cells with the indicated concentration of PTHrP(1-86).

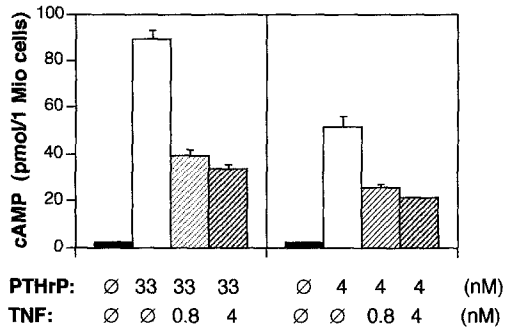

Figure 2. Effect of TNF α on PTHrP-induced cAMP accumulation in fetal rat calvaria cells. The cells were pretreated with vehicle alone (*open bars*) or with TNF α, as in figure 1. The *black bars* represent controls which were not stimulated with PTHrP.

attempted to specifically neutralize this signalling pathway and to evaluate the remaining influence of TNF α on cAMP accumulation. Coincubation of TNF α with H-7, a blocker of PK-C, completely abolished the effect of TNF α on PTHrP-stimulated cAMP accumulation (Fig. 5). H–7 on its own slightly increased intracellular cAMP levels stimulated by PTHrP.

DISCUSSION

TNF α inhibited the PTHrP-stimulated accumulation of intracellular cAMP in osteoblast-like cells. This phenomenon was observed in both a clonal human osteosarcoma cell line

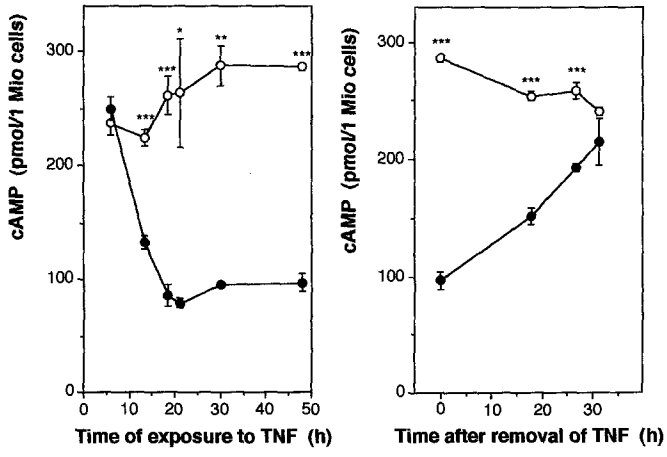

Figure 3. Time course of the inhibitory effect of TNF α on PTHrP-stimulated accumulation of cAMP in Saos-2 cells.
The cells were treated with vehicle alone (control, ○) or with TNF α (10^{-9} M, ●). *Left panel*: Effect of pretreatment with TNF α for 6 to 48 h. *Right panel*: Cells were exposed 24 h to TNF α, washed 3 times, and incubated for the indicated period of time (*x*-axis) in TNF α-free medium. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ (Student t-test).

Table 1. Effect of pertussis toxin on the ability of TNF α to inhibit PTHrP-stimulated accumulation of cyclic AMP in Saos-2 cells

TNF α (pmol/l)	Accumulation of Cyclic AMP (pmol/1 Mio cells)			
	Control	Pertussis toxin (ng/l)		
		4	16	64
Ø	64.6 ± 1.3 (100%)	99.2 ± 3.3 (100%)	92.2 ± 7.5 (100%)	89.8 ± 2.4 (100%)
25	43.2 ± 2.7 (66.9%)	66.7 ± 2.3 (67.2%)	60.9 ± 1.7 (66.1%)	68.4 ± 4.4 (76.2%)
500	34.6 ± 3.3 (53.6%)	49.3 ± 0.2 (49.7%)	47.7 ± 1.1 (51.7%)	44.5 ± 2.3 (49.6%)

Saos-2 cells were pretreated with or without TNF α together with vehicle alone or with Pertussis toxin for 24 h. The relative decrease attributable to TNF α alone is shown in brackets (the PTHrP-stimulated response without TNF α was set to 100 %). Values are the mean ± SD from triplicates.

(Saos-2) and in primary cultures of osteoblast-like cells from fetal rat calvaria. Although we did not measure adenylate cyclase activity directly, it is unlikely that the observed effect was due to an increase in phosphodiesterase activity, since all measurements of intracellular cAMP content were carried out in the presence of high doses of the phosphodiesterase inhibitor isobutylmethyl-xanthine. The inhibition of PTHrP-stimulated accumulation of intracellular cAMP was demonstrable with very low concentrations of TNF α (2×10^{-12} M). The observed inhibition of PTHrP-stimulated cAMP is in agreement with preliminary reports from other groups which have demonstrated similar effects of TNF α on cAMP accumulation stimulated by PTH in the osteoblast-like cell line UMR 106 (20,21).

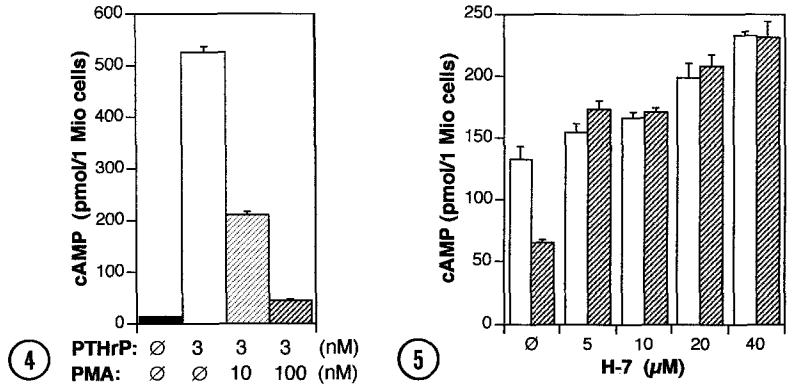

Figure 4. Effect of PMA on the PTHrP-stimulated accumulation of cAMP in Saos-2 cells. The cells were pretreated with vehicle alone (*open bar*) or with PMA for 24 h. The *black bar* represents controls which were not stimulated with PTHrP.

Figure 5. Effect of coincubation with H-7 on the ability of TNF α to inhibit PTHrP-stimulated accumulation of cAMP in Saos-2 cells.
Cells were treated for 24 h with vehicle alone (Ø) or with H-7 (concentrations as indicated on the *x*-axis) in the presence (*hatched bars*) or absence (*open bars*) of TNF α. TNF α (10^{-9} M) was added 1 h after H-7.

Little is known about intracellular signalling pathways activated by TNF α. Modulation of intracellular cAMP, of intracellular calcium, as well as activation of PK-C have been described (4). In both the Saos-2 cells and the fetal rat osteoblasts TNF α did not appear to stimulate adenylate cyclase. The inhibitory effect of TNF α on PTHrP-stimulated cAMP accumulation also did not appear to be due to a direct modulation of PTHrP-induced cAMP formation via a G_i protein, since the effect of TNF α was not influenced by pertussis toxin. Two facts pointed to the involvement of PK-C in the observed inhibition: Firstly, the PK-C stimulator PMA closely mimicked and secondly, the PK-C inhibitor H-7 completely abolished the inhibitory effect of TNF α.

TNF α is known to exert its action either rapidly within minutes in some systems (e. g. (22)), or with a time lag of approx. 24 h in others (23,24). In our study TNF α had no effect on the modulation of PTHrP-stimulated cAMP prior to a exposure for 12 h. This time lag indicates that PK-C activation by TNF α probably had no direct effect on cAMP accumulation but rather that the inhibition was due to secondary events of PK-C on the osteoblast metabolism.

Recent results have shown that inhibition of PTH-stimulated adenylate cyclase by homologous desensitization in UMR 106 cells is mediated by a very similar signalling pathway via PK-C (25). This pathway could be blocked comparably by H-7 and activated by PMA, and resulted in a decrease of the number of PTH receptors. Additionally, modulation of G_i protein function did not influence the mechanism. The only difference compared to TNF α appeared to be the more rapid intracellular signal transmision within 2 h. Whether the downregulation of the PTHrP-induced cAMP stimulation by TNF α might also be due to a heterologous desensitization, will have to be shown in additional studies.

In conclusion, TNF α inhibited PTH/PTHrP-stimulated accumulation of intracellular cAMP time- and dose-dependently in osteoblast-like cells via activation of PK-C. Since TNF α may be released by macrophages as a host response to the presence of the tumor and exerted its effects at concentrations as low as 10^{-12} M, this mechanism may be important for modulating the skeletal effects of PTHrP in the syndrome of humoral hypercalcemia of malignancy.

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