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Regulation of Granulocyte Colony-stimulating Factor and Parathyroid Hormone-related Protein Production in Lung Carcinoma Cell Line OKa-C-1

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Previously we have established a clonal squamous cell carcinoma cell line OKa-C-1 derived from lung cancer of a patient with marked leukocytosis and hypercalcemia. OKa-C-1 cells simultaneously produce granulocyte colony-stimulating factor (G-CSF) and parathyroid hormone-related protein (PTHrP) at the single cell level and cause paraneoplastic syndromes in nude mice bearing the tumor. It is known that the production of G-CSF and PTHrP is individually regulated by inflammatory cytokines in various malignant cells. To investigate the common factors in the regulation of G-CSF and PTHrP production in OKa-C-1 cells, we examined the effects of some inflammatory agents [lipopolysaccharide (LPS), phorbol-12-myristate-13-acetate (PMA), tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1) β and IL-6] on G-CSF and PTHrP production, by means of enzyme-linked immunosorbent assay (ELISA), immunoradiometric assay (IRMA) and quantitative reverse transcription-polymerase chain reaction (RT-PCR). TNF-α or IL-1β induced both G-CSF and PTHrP production in the conditioned medium. TNF-α synergized with IL-1β to significantly increase G-CSF production. In addition, TNF-α and IL-1β strongly induced G-CSF mRNA with peaks at 2 and 6 h respectively. Although PTHrP production was also strongly induced by TNF-α PTHrP mRNA expression was more strongly induced by PMA than by TNF-α. Thus, TNF-α and IL-1β could be common factors that individually and synergistically regulate G-CSF and PTHrP production in OKa-C-1 cells. Moreover, G-CSF and PTHrP production could be not only transcriptionally, but also posttranscriptionally regulated by other factors.

Key words: G-CSF — PTHrP — Lung cancer — OKa-C-1

Some cancer patients are known to manifest both leukocytosis and hypercalcemia.[1–10] The production of granulocyte colony-stimulating factor (G-CSF) or parathyroid hormone-related protein (PTHrP) by cancer cells is thought to be responsible for these paraneoplastic syndromes.[11–16] However, it remains unknown whether simultaneous occurrence of hypercalcemia and leukocytosis is coincidental or not.

G-CSF production is induced in many cell types including monocytes and macrophages,[17] endothelial cells,[18] and fibroblasts by stimulation with endotoxin or lipopolysaccharide (LPS), tumor necrosis factor-α (TNF-α), interferon-γ, interleukin-1 (IL-1), and phorbol-12-myristate-13-acetate (PMA).[19–21] PMA and inflammatory cytokines, such as IL-1α, IL-1β, IL-6, TNF-α, and prostaglandin E2, induce production of PTHrP by synovial fibroblasts in human osteoarthritis.[22] *PTHrP* gene expression is induced in the liver, spleen, heart, lung, and kidney in rats in response to a lethal dose of LPS.[23] Some of these stimuli can also interact with each other or with other agents to influence G-CSF or PTHrP production. For example, retinoic acid synergistically stimulates G-CSF production through the autocrine production of IL-1 in human monocytic leukemic cell line, THP-1.[24] TNF mediates LPS-incduced *PTHrP* gene expression in mouse spleen.[25] Thus, G-CSF and PTHrP are thought to be individually regulated by a complex network of inflammatory cytokines.

We have previously reported the establishment of a human lung squamous cell carcinoma cell line named OKa-C-1 simultaneously producing G-CSF and PTHrP.[7] The aim of the present study was to find common factors simultaneously regulating G-CSF and PTHrP expression in OKa-C-1 cells. We examined the effects of several agents associated with inflammation, such as TNF-α, IL-1β, IL-6, LPS and PMA, on G-CSF and PTHrP production in OKa-C-1 cells using enzyme-linked immunosorbent assay (ELISA), immunoradiometric assay (IRMA) and quantitative reverse transcription-polymerase chain reaction (RT-PCR).

MATERIALS AND METHODS

Reagent and supplies *Escherichia coli* strain O111:B4 endotoxin (LPS), PMA, recombinant human TNF-α, IL-1β and IL-6 were from Sigma (St. Louis, MO). LPS and PMA were prepared in phosphate-buffered saline and dimethyl sulfoxide (DMSO), respectively, as 1000× stock

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solutions and were handled in subdued light. ELISA kits for measuring the contents of G-CSF and PTHrP in culture medium were from TECHNE Corp. (Minneapolis, MN) and Mitsubishi Chemical Corp. (Tokyo), respectively.

Cell culture We previously established a nude mouse transplantable cell line, OKa-N-1, from a lung squamous cell carcinoma in a patient with both hypercalcemia and leukocytosis. We also established an *in vitro* clonal cell line, OKa-C-1, from this tumor cell line. The serum levels of both G-CSF and PTHrP are elevated in nude mice transplanted with OKa-C-1 as well as OKa-N-1. OKa-C-1 cells produce both G-CSF and PTHrP in culture, and express both G-CSF and PTHrP mRNA. The OKa-C-1 cells were cultured in RPMI-1640 medium containing 10% fetal calf serum (FCS) at 37°C in an incubator, under a 100% humidified 5% CO_2 atmosphere. Subcultures were carried out twice weekly by removing the cells from 75 cm^2 tissue culture flasks with 0.025% trypsin in 0.02% EDTA and splitting them 1:3. For experiments, all cultures were grown to confluency in 24-well tissue culture plates and 25 cm^2 tissue culture flasks in the presence of 10% FCS. Confluent cultures were rinsed with serum-free medium and 1% FCS-containing medium was conditioned for 24 h. The medium was replaced with fresh medium containing 1% FCS. The cells were treated with either LPS, PMA, TNF-α, IL-1β, IL-6 or combinations thereof under the conditions indicated in the figure legends. Each conditioned medium contained DMSO and phosphate-buffered saline at the same final concentrations as the control. After 72 h, cell culture medium was collected, centrifuged at 1000*g* for 10 min to remove cell debris and stored at −80°C. For RNA analysis, cells were harvested with a cell scraper (Beckton Dickinson, Lincoln Park, NJ) at the times indicated in the figure legends. Each experiment was performed in triplicate.

DNA extraction and Southern blot analysis The surgically resected OKa-N-1 tumors were treated with proteinase K and extracted with phenol and chloroform. In our previous report, we showed that mice with OKa-N-1 tumors have significantly elevated serum levels of G-CSF and PTHrP in addition to marked leukocytosis and hypercalcemia. DNA of peripheral blood mononuclear cells (PBMCs) provided by a healthy donor was extracted as a control DNA. Purified DNA (10 μg) was digested with *Eco*RI or *Hin*dIII (Toyobo, Tokyo), electrophoresed through 1% agarose gels, and transferred onto nylon membranes (Bio-Rad, Richmond, CA). The nylon membranes were hybridized to a ^{32}P-labeled human G-CSF or PTHrP cDNA probe (kindly provided by Chugai Pharmaceutical Co., Tokyo). Hybridization was performed for 16 h at 42°C in 40% formamide, 4× standard sodium citrate (SSC), 20 mmol/liter Tris HCl (pH 8.0), 1× Denhardt's solution, 0.1% sodium dodecyl sulfate (SDS) and 10% dextran sulfate. Blots were washed twice for 15 min in 0.1% SDS/0.1× SSC at 68°C and autoradiographed for 24 h at −80°C using intensifying screens.

Detection of G-CSF and PTHrP G-CSF and PTHrP contents in the culture medium were determined using ELISA (Genzyme-TECHNE Research Products, Minneapolis, MN) and immunoradiometric assay (Mitsubishi Chemical Co., Ibaraki), respectively, according to the supplier's instructions. The sensitivities of these kits are 0.4 pg/ml for G-CSF and 1 pmol/liter for PTHrP. G-CSF and PTHrP levels in the fresh medium containing 1% FCS were both undetectable with these kits.

Quantitative RT-PCR Total RNA was extracted from the cultured cells exposed to various agents as indicated in the figure legends by the guanidine-thiocyanate method using ISOGEN solution (Nippon Gene Co., Tokyo). One microgram of the RNA was converted to cDNA with Molony murine leukemia virus reverse transcriptase in 20 μl of reaction mixture. For quantification, 1 μl aliquots of cDNA samples were subjected to PCR in 50 μl of reaction solution containing 0.5 μ*M* of each specific primer, 10 m*M* Tris-HCl, 50 m*M* KCl, 1.5 m*M* $MgCl_2$, 0.2 m*M* dNTP, and 1 unit of *Taq* polymerase. Primers used for G-CSF were 5′-TAGAGCAAGTGAGGAAGATCCAGG-3′ for sense and 5′-AGTTCTTCCATCTGCTGCCAGATG-3′ for antisense, giving a 328 bp fragment. Primers used for PTHrP were 5′-GCGACGATTCTTCCTTCACC-3′ for sense and 5′-AGAGTCTAACCAGGCAGAGC-3′ for antisense, yielding a 285 bp fragment. Primers used for β-actin were 5′-ACCTTCAACACCCCAGCCATG-3′ for sense and 5′-GGCCATCTCTTGCTCGAAGTC-3′, giving a 309 bp fragment. The reaction was performed for 20 cycles for β-actin and 32 cycles for G-CSF and PTHrP in a DNA thermal cycler (TaKaRa, Ohtsu). Each PCR cycle involved denaturation at 94°C for 1 min, annealing at 58°C for 1 min, and polymerization at 72°C for 1 min. Amplification cycle numbers were optimized for each sequence using the serial dilution method to achieve a dose-dependent amplification.[26] One microliter of PCR product was electrophoresed on 1% NuSieve GTG agarose gel and stained with ethidium bromide. The intensity of the bands was evaluated using a UV-light box imaging system (Atto, Tokyo).

RESULTS

Southern blotting analysis of *G-CSF* and *PTHrP* genes In order to see if the constitutive expression of G-CSF and PTHrP by the tumor cells is due to changes in genomic structure, we carried out Southern blot analysis. DNA (10 μg) isolated from OKa-N-1 tumor cells and normal PBMC was digested with *Eco*RI or *Hin*dIII, and electrophoresed on 1% agarose gel. After being transferred to nylon membrane, they were hybridized to G-CSF and PTHrP cDNA probes. The sizes of bands detected with OKa-N-1 DNA

were identical to those with PBMC DNA, regardless of the restriction enzyme used (Fig. 1). These results are consistent with the reported restriction maps of the *G-CSF* and *PTHrP* genes.[21, 27] Moreover, there was little difference in signal intensity between OKa-N-1 and PBMC DNA. Thus, the *G-CSF* and *PTHrP* genes of OKa-N-1 cells appeared to have no rearranged configuration or amplification.

Effect of cytokines on G-CSF or PTHrP production by OKa-C-1 cells OKa-C-1 cells were treated with vehicle control, LPS, PMA, TNF-α, IL1-β and IL-6 in RPMI-1640 medium containing 1% FCS. Culture medium was collected at 72 h and assayed for G-CSF content by ELISA. The concentration-response curves were obtained with each agent (Fig. 2a). G-CSF content in the medium cultured with vehicle control for 72 h was undetectable with this kit. In this study, the ability of the cells to constitutively produce G-CSF seemed to be on the decline during several passages. Constitutive G-CSF production was 3±2 pg/ml. IL-1β or TNF-α significantly stimulated G-CSF production. Maximal induction of G-CSF production by IL-1β and TNF-α was observed at concentrations between 1.0 to 20 and 200 to 400 ng/ml, respectively. TNF-α increased G-CSF level almost five times more than IL-1β. We next examined the synergistic effects of the two different agents on G-CSF production by OKa-C-1 cells. OKa-C-1 cells were treated with various combinations LPS, PMA, TNF-α, IL1-β and IL-6 (20 ng/ml). The con-

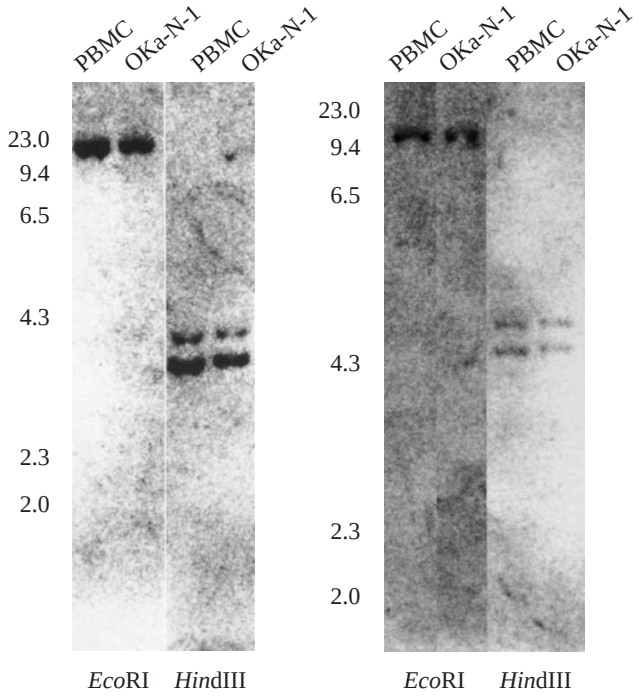

Fig. 1. Southern blot analysis of the *G-CSF* and *PTHrP* genes from OKa-N-1 cells and peripheral blood mononuclear cells (PBMC). DNA (10 μg) from OKa-N-1 cells and normal PBMCs were cleaved with *Eco*RI or *Hin*dIII. Cleaved DNA was hybridized with G-CSF (A) and PTHrP (B) cDNA probes.

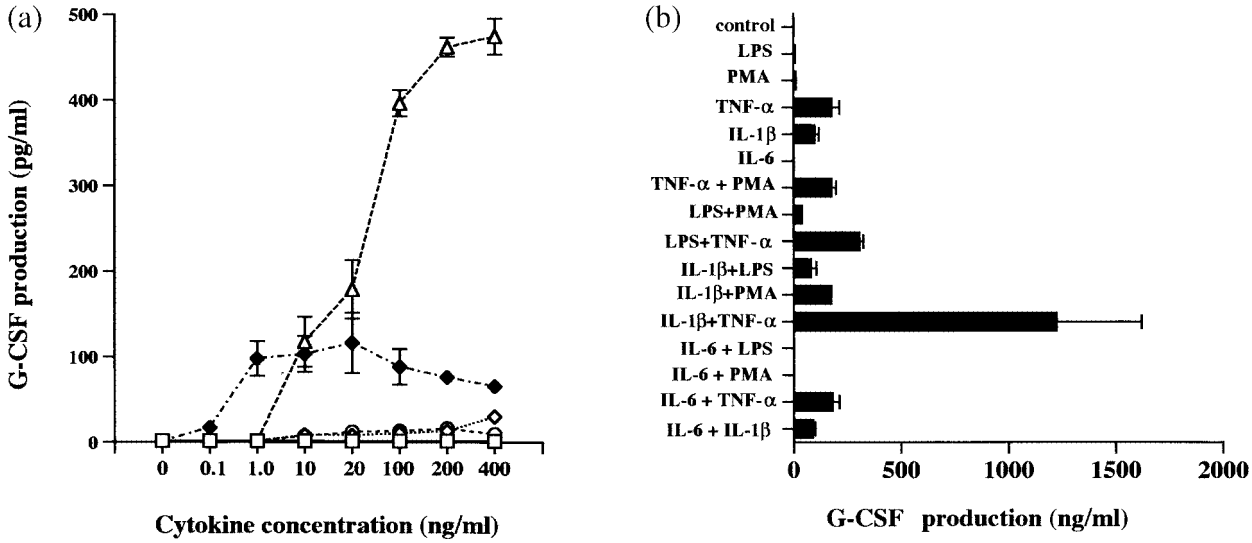

Fig. 2. Effects of LPS, PMA, TNF-α, IL-1β and IL-6 on G-CSF (a) and PTHrP (b) production by OKa-C-1 cells. On day 3 after plating, confluent cells were rinsed three times with medium without serum and 1% FCS-containing medium conditioned for 24 h. The cell layer was rinsed and incubated with various concentrations of the individual agents (G-CSF, from 0 to 400 ng/ml; PTHrP, 20 and 200 ng/ml) in the presence of 1% FCS for 3 days. Culture medium was collected then assayed for G-CSF and PTHrP content by ELISA and radioimmunoassay, respectively. The data are the mean±SD of three experiments. Fig. 2a: □, control; ◇, LPS; ○, PMA; △, TNF-α; ■, IL-6; ◆, IL-1β. Fig. 2b: □, 20 ng/ml; ■, 200 ng/ml.

ditioned medium was assayed for G-CSF content 72 h after the start of incubation (Fig. 2b). LPS and IL-1β synergized with TNF-α to stimulate G-CSF production.

The combination of TNF-α with IL-1β increased G-CSF level about sixfold as much as TNF-α alone. PTHrP content in the culture medium was assayed with an immunoradiometric assay kit. PTHrP production by OKa-C-1 cells was observed at two different concentrations (20 and 200 ng/ml) of individual agents (Fig. 3a). Constitutive PTHrP production was 12±2 pmol/liter in OKa-C-1 cells. LPS, PMA, TNF-α and IL1-β increased the PTHrP level 1.5±2-fold, 2.0±3-fold, 3.5±4-fold and 1.6±1-fold over the control, respectively. LPS stimulated PTHrP production at the concentration of 200 ng/ml while PMA, TNF-α and IL1-β did so at both concentrations. There was little difference in the extent of PTHrP production at concentrations between 20 and 200 ng/ml of individual agents except for LPS. No combination of the various agents had a greater effect than TNF-α alone on PTHrP production (Fig. 3b).

Effect of cytokines on G-CSF or PTHrP mRNA expression OKa-C-1 cells treated with various cytokines were harvested at the periods indicated in the figure legends. The expression of G-CSF and PTHrP mRNA in the cells was detected by quantitative RT-PCR. The time courses of G-CSF and PTHrP mRNA induction by individual agents at a concentration of 20 ng/ml are shown in Fig. 4a. The

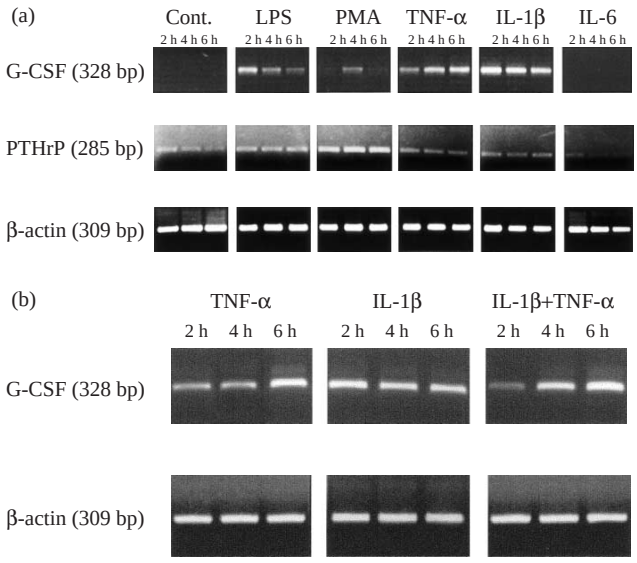

Fig. 4. RT-PCR for assessing the change in the expression of mRNA for G-CSF and PTHrP in 2, 4 and 6 h cultures of OKa-C-1 cells. The cells were treated with vehicle, LPS, PMA, TNF-α, IL-1β or IL-6 (a). The G-CSF mRNA induction by a combination of TNF-α with IL-1β was compared with that by the two agents individually (b). The concentrations of agents were: LPS, 20 μg/ml; PMA, 20 μg/ml; TNF-α, 20 μg/ml; IL-1β, 20 μg/ml and IL-6, 20 μg/ml.

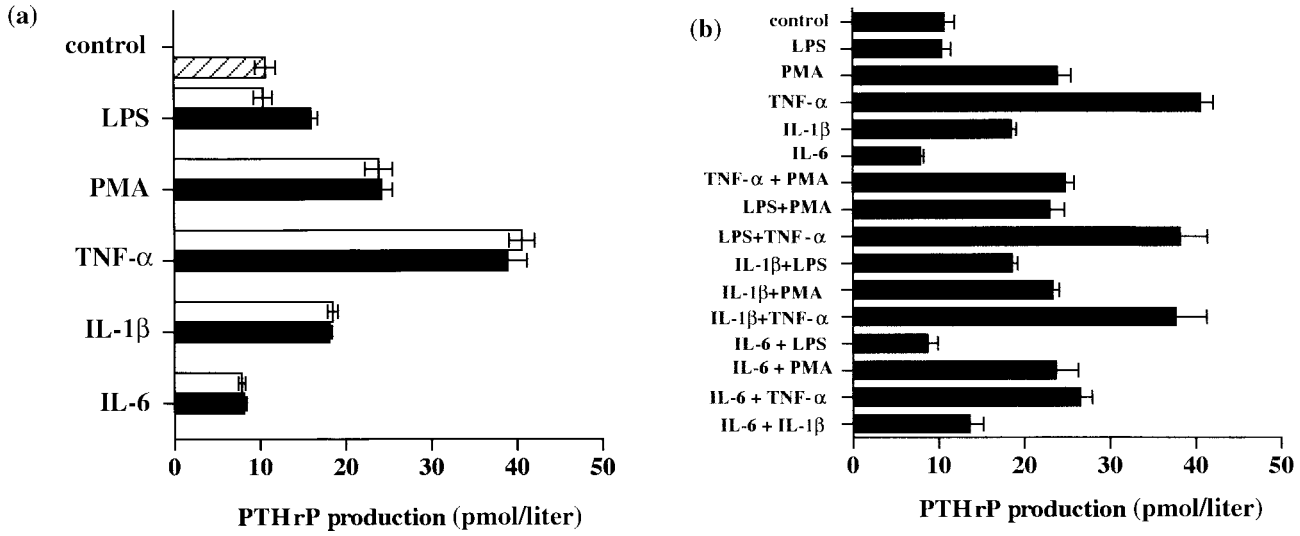

Fig. 3. Effects of two different agents in combination on G-CSF (a) and PTHrP (b) production by OKa-C-1 cells. On day 3 after plating, confluent cells were rinsed three times with medium without serum and 1% FCS-containing medium conditioned for 24 h. The cell layer was rinsed and incubated with the indicated combinations in the presence of 1% FCS for 3 days. Culture medium was collected and subsequently assayed for G-CSF and PTHrP content by ELISA and radioimmunoassay, respectively. The concentrations of agents were: LPS, 20 μg/ml; PMA, 20 μg/ml; TNF-α, 20 μg/ml; IL-1β, 20 μg/ml and IL-6, 20 μg/ml. The data are the mean±SD of three experiments.

constitutive production of G-CSF transcripts seemed to be on the decline during several passages. The maximum increase of G-CSF mRNA expression induced by LPS and IL-1β was observed at 2 h. IL-1β induced G-CSF mRNA expression for a longer time than LPS. The maximum increases of G-CSF mRNA induced by PMA and TNF-α were observed at 4 and 6 h, respectively. IL-6 did not induce G-CSF mRNA expression. Other agents were less potent than PMA in inducing PTHrP mRNA expression. An increase of PTHrP mRNA expression by PMA occurred at 2 h and was maintained for 6 h. Next we studied whether the combinations, which synergistically stimulated G-CSF production, could likewise induce G-CSF mRNA expression. The maximum increase of G-CSF mRNA expression by the combination of TNF-α with LPS or IL-1β was observed at 6 h. G-CSF mRNA expression induced by the combination of TNF-α and IL-1β was higher than that induced by each cytokine alone (Fig. 4b). The expression of G-CSF and PTHrP mRNA was no longer induced by any agent or combination at 12 h (data not shown).

DISCUSSION

Occasionally, leukocytosis and hypercalcemia are simultaneously observed in patients who have squamous cell carcinoma of the oral cavity, lung, head or neck with an aggressive clinical course.[1, 2, 28, 29] The production of G-CSF and PTHrP by cancer cells is thought to be responsible for these paraneoplastic syndromes. However, the mechanism of simultaneous and unrestricted production of G-CSF and PTHrP by cancer cells is not well delineated.

Leukocytosis has been reported in nonhematologic malignancies with an incidence of 14.7 to 30%, and is more prevalent (35%) among lung cancer patients.[30] Hypercalcemia also occurs frequently in squamous cell carcinoma of the lung.[13] We have previously established an *in vivo* cell line OKa-N-1 and an *in vitro* clonal cell line OKa-C-1, originally derived from a squamous cell lung carcinoma patient with both leukocytosis and hypercalcemia. OKa-C-1 cell line exhibits continuous expression of both G-CSF and PTHrP at the single cell level, and should be an useful tool for studying the mechanism of simultaneous production of G-CSF and PTHrP.

Gene amplification is one possible mechanism for the over-expression of a specific gene.[31, 32] However, Southern blotting analysis did not show any amplification or rearrangement of the *G-CSF* and *PTHrP* genes in OKa-N-1 cells. Further, chromosomal aberrations and gene amplification of G-CSF or PTHrP are not necessarily associated with over-expression of G-CSF or PTHrP.[8, 32, 33] These data suggest the existence of *trans*-acting regulatory mechanisms which turn on the transcription of *G-CSF* and *PTHrP* genes.

Autonomous G-CSF or PTHrP production is occasionally detected in malignant neoplasms without apparent infection.[8, 34] However, it has been reported that several inflammatory cytokines, which are produced by host immune cells or tumor cells themselves, could influence the production of G-CSF and PTHrP during the course of malignant disease.[28] Indeed, Rizzoli *et al*. have shown that PMA, IL-6 and TNF-α induced an increase in PTHrP production by a human lung squamous cell carcinoma line, which was associated with elevated levels of PTHrP mRNA.[35] G-CSF is also produced in many cell types including monocytes and macrophages,[17] endothelial cells,[18] and fibroblasts[36] upon stimulation with endotoxin, LPS, TNF-α, interferon-γ, or IL-1.[19, 20, 37]

In this study, we examined which inflammatory agents regulate simultaneous production of G-CSF and PTHrP in the OKa-C-1 cell line. We showed that TNF-α and IL-1β increased G-CSF production in the human lung squamous cell carcinoma line OKa-C-1. The maximum increase of G-CSF mRNA expression induced by IL-1β and TNF-α was observed at 2 and 6 h, respectively. G-CSF mRNA expression induced by IL-1β was well maintained even after 6 h, while that induced by LPS peaked at 2 h then declined quickly. TNF-α induced G-CSF production in cultured medium about twice as effectively as IL-1β. G-CSF in the culture medium was almost unaffected by LPS. These results suggest that the G-CSF level in the culture medium could depend on the extent and duration of G-CSF mRNA expression. LPS or IL-1β synergized with TNF-α to stimulate G-CSF production. The combination of IL-1β and TNF-α significantly increased G-CSF production at both the protein and mRNA levels. With this combination, G-CSF mRNA expression was slightly induced at 2 h and maximally induced at 6 h, suggesting that IL-1β may enhance the effect of TNF-α on G-CSF mRNA expression. TNF-α induced PTHrP production most intensely. However, PTHrP mRNA expression was induced most strongly by not TNF-α, but PMA. Thus, posttranscriptional factors may participate in PTHrP production in OKa-C-1 cells. In our study, IL-6 and PMA appeared to inhibit TNF-α induced PTHrP production, but it is not clear whether these effects are significant or not. The regulation of PTHrP production by IL-6 and PMA may differ according to the cell origin or phenotype.

Yoneda *et al*. demonstrated that splenic cytokines such as tumor necrosis factor, which are produced by normal host cells stimulated by the presence of the tumor, could influence the development of hypercalcemia, leukocytosis and cachexia.[28] IL-1β considered to be a major osteoclast-activating factor, increases the concentration of serum calcium synergistically with PTHrP.[38] Thus, TNF-α and IL-1β may play important roles in hypercalcemia and leukocytosis. In this study, we demonstrated that TNF-α and IL-1β could act to increase both G-CSF and PTHrP produc-

tion at the single cell level in the OKa-C-1 cell line. However, our results showed that other factors besides TNF-α and IL-1β also participate in PTHrP and G-CSF production. Further studies are required to elucidate the complex cytokine network regulating PTHrP and G-CSF production.

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