

THE stimulation of granulocyte macrophage-colony stimulating factor (GM-CSF) by interleukin-1 (IL-1) has been shown to be counteracted in different mesenchymal cell systems by cyclic adenosine monophosphate (cAMP) agonists. The aim of this study was the evaluation of different cAMP agonists on GM-CSF expression in human bone marrow stromal cells. Incubation of secondary haematopoietic progenitor cell deprived human stromal cell cultures with IL-1 or TNF- α induced GM-CSF protein expression in culture supernatants and GM-CSF-mRNA in adherent stromal cells. The coincubation with 8-bromo-cAMP (8BrcAMP), a water soluble cAMP analogue, inhibited this GM-CSF stimulation at the protein and the mRNA level. This effect was dose dependent with a maximal inhibition of about 65% occurring at a 8BrcAMP concentration of 0.75 mM. In addition to 8BrcAMP, other cAMP agonists such as dibutyryl-cAMP, forskolin, pertussis toxin, or prostaglandin E₂ (PGE₂) had the same inhibitory effect on GM-CSF stimulation by IL-1. Coincubation with the cyclooxygenase inhibitor indomethacin had no significant influence on GM-CSF expression in stromal cells. Our results provide evidence that the previously described inhibitory effect of cAMP agonist PGE₂ on haematopoietic progenitor cells *in vivo* is, at least in part, mediated by modulating the expression of GM-CSF in bone marrow stromal cells.

Key words: GM-CSF; bone marrow stromal cells, c-AMP, IL-1

cAMP analogues downregulate the expression of granulocyte macrophage colony-stimulating factor (GM-CSF) in human bone marrow stromal cells *in vitro*

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Introduction

The bone marrow stroma provides a microenvironment for proliferation and differentiation of haematopoietic stem and progenitor cells. Stromal cell cytokine production plays a pivotal role in controlling the development of sufficient mature blood cells under normal demands or after stimulation by inflammatory processes.¹ GM-CSF is a potent growth factor for a variety of haematopoietic progenitor cells. It is produced by T lymphocytes, macrophages and a variety of mesenchymal cells.²

Long-term bone marrow Dexter type cultures support the prolonged proliferation of haematopoietic progenitor cells, thereby providing an *in vitro* model for studies aimed at elucidating the mechanisms involved in haematopoietic regulation. The adherent layer of Dexter cultures is composed of mesenchymal bone marrow stromal cells.³ The regulation of growth factor production in the adherent layer of Dexter type long-term bone marrow cultures closely resembles the *in vivo* situation. Multiple cytokines have been found in the supernatant of Dexter cultures, including GM-CSF, G-CSF, M-CSF, interleukin-6, and leukaemia inhibitory factor (LIF). GM-CSF expression in mesenchymal cells is induced

by inflammatory cytokines like IL-1 or TNF- α and is controlled at both the transcriptional and post-transcriptional levels.^{4–6}

In this study, we used human stromal cell cultures to evaluate the effects of cAMP on the expression of GM-CSF, induced by inflammatory cytokines. We demonstrated that a variety of cAMP agonists inhibit the production of GM-CSF at the protein and mRNA level. These data indicate that the inhibitory effect of cAMP agonists like PGE₂ on haematopoietic progenitor cells are, at least in part, mediated by modulating the expression of growth factors like GM-CSF in the bone marrow microenvironment.

Materials and Methods

Material

rh-IL-1 α was kindly provided by Hoffmann La Roche (Nutley, NJ), and rh-TNF α (6.6×10^6 U/mg) by Knoll AG (Ludwigshafen, Germany). Indomethacin, pertussis toxin, dibutyryl-cAMP, 8BrcAMP, PGE₂, and forskolin were purchased from Sigma Chemicals (Deisenhofen, Germany), and α -³²P-labelled nucleotides from Amersham Buchler (Braunschweig, Germany).

Stromal cell culture

Adherent bone marrow stromal cell cultures were essentially obtained as previously described.⁷ Bone marrow mononuclear cells, separated by centrifugation over Ficoll-Hypaque, were incubated at a cell density of 1×10^6 /ml in 25 cm² tissue flasks at 33°C in culture medium consisting of RPMI 1640 supplemented with 10% fetal calf serum (FCS), 10% horse serum, 1.0 μM hydrocortisone (Sigma Chemicals), and the additives as described.⁷ In weekly intervals, cultures were fed by replacing 75% of the culture medium. When cultures were covered more than 80% by adherent cells, the primary cultures were treated with trypsin-EDTA (Biochrom, Berlin, Germany). Detached stromal cells were pooled and expanded in new culture flasks at a surface ratio of 1:5 in 75 cm² tissue flasks. The adherent cells were incubated under the same culture conditions until the cultures became confluent again. By this culture method, homogeneous stromal cell layers were obtained that were morphologically and functionally comparable in different culture flasks. These cultures were devoid of all haematopoietic cells, including macrophages. Hydrocortisone was removed from these cultures at least 3 days before use for RNA and protein analysis. GM-CSF was measured in the culture supernatants of stromal cells after incubation for 24 h with various factors, as indicated in the Results section. For RNA analysis, the factors, as indicated in the Results section, were added simultaneously to parallel culture flasks from the same donor. After 6 h the cells were lysed directly in the culture flask with guanidium-isothiocyanate lysis buffer and processed as described below. The content of a 75 cm² tissue flask yielded 10–20 μg total RNA.

Northern blot analysis

Total cytoplasmic RNA was prepared using the single step method of guanidium/phenol-chloroform extraction as described earlier.⁸ Ten to 15 μg of RNA (depending on the least yield obtained in each experiment) were subjected to electrophoresis on a 1% agarose-formaldehyde gel and transferred onto nylon membrane (Hybond-N, Amersham Buchler). Blots were hybridized to α^{32} P-labelled cDNA probes using random primer DNA labelling kit (Boehringer), washed and exposed to Cronex-4 autoradiography films (DuPont) at -70°C. Human GM-CSF cDNA, an 800-bp EcoRI fragment cloned into P91023(B)-vector, was obtained from American Type Culture Collection (Rockville, MD).

Enzyme-linked immunosorbent assay (ELISA)

Concentrations of GM-CSF were measured using Quantikine Human Cytokine Immunoassays (R&D Systems, Minneapolis, MN) according to manufacturer's

instructions. Sensitivity of the test for human GM-CSF was 7.8 pg/ml.

Results

Stimulated GM-CSF expression in human bone marrow stromal cells is inhibited by 8BrcAMP

Expression of GM-CSF in human bone marrow stromal cells was assessed at the protein level in culture supernatants by a sensitive ELISA and at the mRNA level by Northern blot analysis. In unstimulated cultures no or very low levels of GM-CSF mRNA were detected. After incubation with IL-1 (100 U/ml) or TNF- α (500 U/ml) for 6 h, significant GM-CSF-mRNA expression was induced. IL-1 was shown to have a stronger stimulatory effect than TNF- α (Figs 1 and 2B). These RNA-data were confirmed at the protein level. IL-1 and TNF- α incubation for 24-h induced GM-CSF secretion into stromal cell culture supernatants with

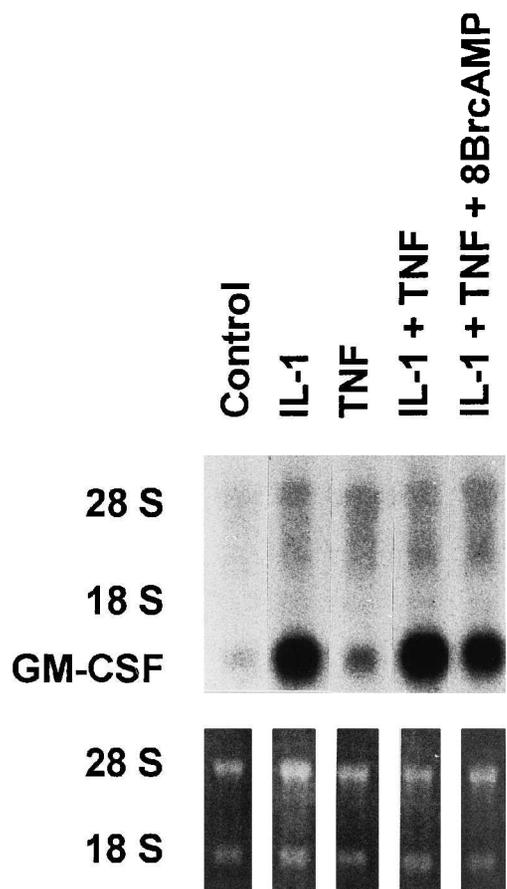
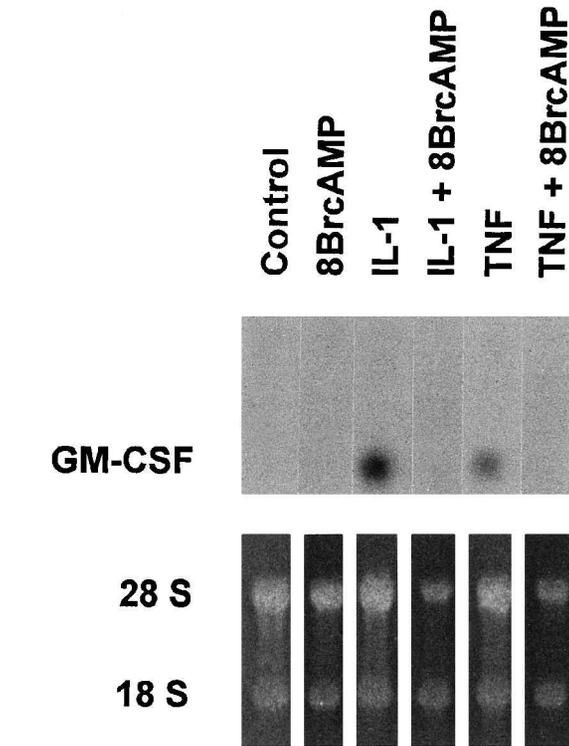
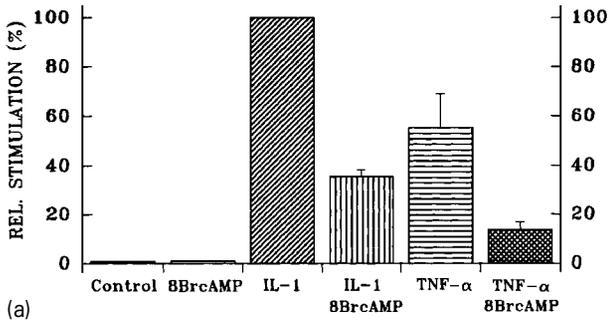


FIG. 1. Induction of GM-CSF mRNA in human bone marrow stromal cells. Confluent secondary stromal cultures were incubated for 6 h in medium alone or with the addition of IL-1 (100 U/ml), TNF- α (500 U/ml), IL-1 plus TNF- α with or without 8BrcAMP (0.5 mM). Total cellular RNA was prepared and subjected to Northern-blot analysis with cDNA probe for human GM-CSF. The 18 and 28S rRNA from ethidium bromide stained gel is shown as loading control. Data are representative for two experiments with stromal cells from different donors.



(a)

(b)

FIG. 2. Effect of 8BrcAMP on the expression of GM-CSF by human stromal cells. Confluent secondary stromal cultures were incubated in medium alone or with the addition of IL-1 (100 U/ml), TNF- α (500 U/ml), 8BrcAMP (0.5 mM) or combinations as indicated. (A) After incubation for 24 h, GM-CSF concentrations in the supernatants were assessed using a specific ELISA. GM-CSF levels are presented as deviation from maximal stimulation. Single experiments were carried out in triplicate, and data from three independent experiments are summarized as mean \pm SEM. (B). After incubation for 6 h, total cellular RNA was prepared and subjected to Northern-blot analysis with cDNA probe for human GM-CSF. The 18 and 28S rRNA from ethidium bromide stained gel is shown as loading control. Data are representative for three experiments with stromal cells from different donors.

IL-1 again being the stronger stimulus. Because the absolute amount of GM-CSF expression varied between experiments, the protein level in culture supernatants from pooled experiments is depicted in Fig. 2A relative to the maximal stimulation.

The addition of the cAMP agonist 8BrcAMP to stromal cell cultures, stimulated by IL-1, TNF- α or the combination of IL-1 and TNF- α , resulted in sig-

nificantly reduced GM-CSF mRNA expression. Similarly, the GM-CSF protein secretion, induced by IL-1 or TNF- α , was inhibited with 8BrcAMP (0.5 mM) cocubation by $65.5 \pm 2.9\%$ or $61.6 \pm 6.1\%$ respectively. These results were obtained by pooling data from three independent experiments (Figs 1 and 2). Dose dependence of the modulatory effect of 8BrcAMP on GM-CSF secretion was evaluated at concentrations between 0.1 and 1.0 mM. Significant inhibition of IL-1 stimulated GM-CSF secretion was observed at 8BrcAMP concentrations of 0.1 to 0.25 mM and the maximal inhibition was seen at concentrations between 0.75 and 1 mM (Fig. 3).

Different cAMP agonists inhibit GM-CSF expression in stimulated human bone marrow stromal cells

In order to evaluate whether the results obtained by 8BrcAMP incubation of human bone marrow stromal cells on GM-CSF expression could be generalized to other cAMP agonists we tested a panel of substances. IL-1 stimulated GM-CSF protein secretion into stromal cell supernatants was inhibited by all investigated cAMP agonists including forskolin at 10 μ M (81.6 ± 5.8 inhibition), dibutyryl-cAMP at 1 mM ($66.1 \pm 8.1\%$ inhibition), pertussis toxin at 1 μ g/ml ($59.0 \pm 4.6\%$ inhibition), and PGE₂ 1 μ g/ml ($36.4 \pm 14\%$ inhibition). These data were confirmed by Northern blot analysis at the mRNA level. The cocubation of stromal cell cultures stimulated by IL-1 with forskolin, PGE₂, dibutyryl-cAMP, and pertussis toxin led to abolishment of GM-CSF mRNA expression. On the other hand, the cyclooxygenase inhibitor and indirect cAMP antagonist indomethacin had no significant

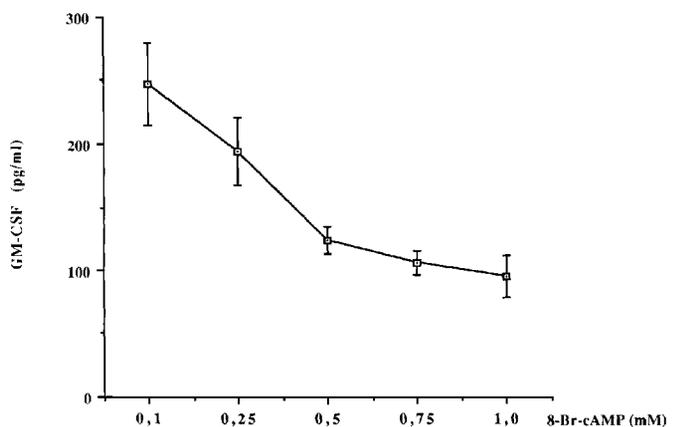


FIG. 3. Dose-dependents of the inhibitory effect of 8BrcAMP on the synthesis of GM-CSF in stromal cells. Confluent secondary stromal cultures were incubated for 24 h in medium containing IL-1 (100 U/ml) with increasing concentrations of 8BrcAMP ranging from 0.1 to 1 mM. The GM-CSF concentration in the supernatants was assessed using a specific ELISA. Experiments were carried out in triplicate, and data from three independent experiments are summarized as mean \pm SEM.

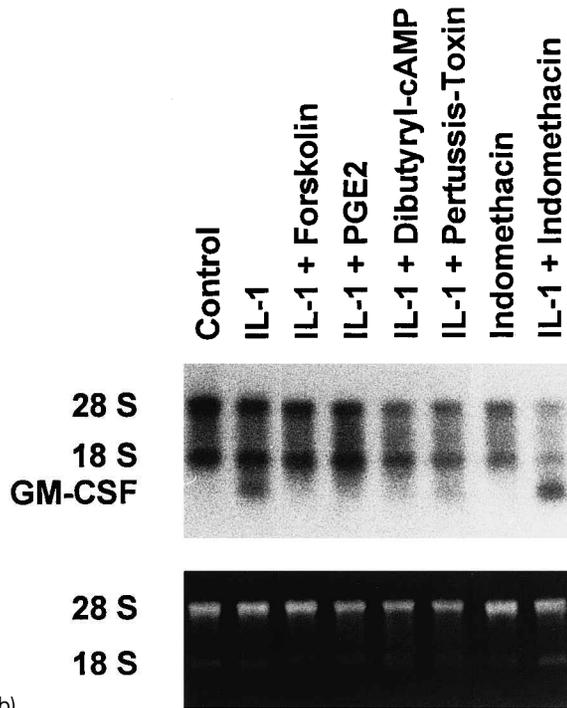
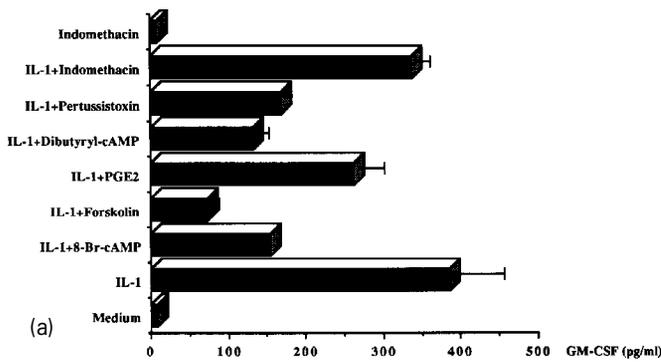


FIG. 4. Effect of different cAMP antagonists on the expression of GM-CSF by human stromal cells. Confluent secondary stromal cultures were incubated in medium alone or with the addition of the indicated stimuli. Concentrations used: IL-1 (100 U/ml), forskolin (10 μ M), PGE₂ (1 μ M), dibutryl-cAMP (1 mM), pertussis toxin (1 μ g/ml), indomethacin (10 μ M). (A) After incubation for 24h, GM-CSF concentrations in the supernatants were assessed using a specific ELISA. GM-CSF levels are presented as deviation from maximal stimulation. Single experiments were carried out in triplicate, and data from three independent experiments are summarized as mean \pm SEM. (B) After incubation for 6h total cellular RNA was prepared and subjected to Northern-blot analysis with cDNA probe for human GM-CSF. The 18 and 28S rRNA from ethidium bromide stained gel is shown as loading control. Data are representative for two experiments with stromal cells from different donors.

inhibitory influence on GM-CSF protein or mRNA expression (Fig. 4).

Discussion

The presented data show that GM-CSF is produced in adherent human bone marrow stromal cell cultures after stimulation with inflammatory cytokines IL-1

and TNF- α . This effect has previously been reported by several other groups.⁹⁻¹¹ Some authors also described the endogenous expression of GM-CSF in the adherent layer of human Dexter-type cultures without external cytokine stimulation. GM-CSF mRNA could until now unequivocally only be demonstrated by PCR technique in stromal cells cultures.^{9,10,12} Charbord *et al.*¹³ showed the inhibition of GM-CSF activity in complete Dexter-type cultures by neutralizing monoclonal antibodies that suppressed 70% of the endogenous CFU-GM. In our culture system, we could not demonstrate significant production of GM-CSF in human stromal cell cultures by a sensitive ELISA. This fact could be due to the deprivation of haematopoietic cells. These cells are likely to exert a paracrine stimulatory effect on adherent mesenchymal stromal cells and explain the constitutive expression of GM-CSF in complete Dexter-type cultures.

Both IL-1 and TNF- α were able to induce GM-CSF in bone marrow stromal cell cultures, with IL-1 being the more potent inducer. These results are in accordance with published data.¹¹

In a variety of investigated systems, it has been shown that incubation with cAMP agonists causes a differential effect on the production of cytokines. While some cytokines, such as interleukin-6 and leukaemia inhibitory factor, are induced by increased intracellular cAMP concentrations, the expression of other cytokines is inhibited.¹⁴⁻¹⁶ To the latter group belong macrophage-colony stimulating factor, IL-1, platelet derived growth factor, and GM-CSF.¹⁷⁻¹⁹ Patil and Borch reported on the inhibitory role of PGE₂ on GM-CSF production in human fibroblasts. This effect was mediated by increased intracellular cAMP levels. Our group has described a similar finding of PGE₂ and other cAMP agonists inhibiting GM-CSF expression in a murine bone marrow stromal cell line.^{20,21} The present data proves the same inhibitory effect of increased intracellular cAMP concentration in human bone marrow stromal cells at the protein and mRNA level. This inhibition was dose-dependent as shown by increasing suppression of IL-1-induced GM-CSF protein production by the water-soluble cAMP analogue 8BrcAMP. Maximal inhibition of about 60% was obtained at a concentration of 0.75 mM. The inhibitory action of 8BrcAMP was demonstrated on GM-CSF expression induced by IL-1, TNF- α , or the combination of IL-1 and TNF- α . The interference with the stimulatory action of different cytokines indicates that cAMP interacts with components that are either shared among different signalling pathways or act downstream from the convergence point of these pathways. However in earlier experiments on the murine bone marrow stromal cell line +/-1.LDA11 we demonstrated that IL-1 and TNF- α incubation had no influence on intracellular cAMP levels.²⁰

In addition to the water soluble cAMP analogues 8BrcAMP and dibutryl-cAMP other cAMP agonists

like forskolin, PGE₂ or pertussis toxin were shown to inhibit GM-CSF expression. PGE₂ is known to increase the intracellular cAMP synthesis in mesenchymal bone marrow cells.²² In contrast to data obtained by Hamilton *et al.*²³ in synovial fibroblasts our data did not show an additive effect of the cyclooxygenase inhibitor indomethacin on the IL-1 stimulated GM-CSF induction. This discrepancy may be related to different levels of cyclooxygenase activity in different cellular systems. A regulatory loop has been described by which IL-1 induced GM-CSF expression is down-regulated later by IL-1 induced PGE₂.²⁴ Prostaglandins are known to exert an inhibitory action on proliferation of haematopoietic progenitor cells.^{25–27} The mechanism of this effect is not completely understood yet. It seems likely that this inhibition, at least in part, is mediated by a decreased expression of haematopoietic growth factors like GM-CSF by accessory cells.

The mechanism of action by which cAMP decreases GM-CSF expression in stromal cells has been evaluated by our group in a murine bone marrow stromal cell line before. Cyclic-AMP was found to induce the synthesis of a protein which in turn decreased GM-CSF-mRNA stability.²¹ The same mechanism of action has been reported in human lung fibroblasts.²⁴ Overall the present data provide evidence for the previously described inhibitory effect of the second messenger cAMP on the GM-CSF expression occurring in human bone marrow stromal cells. This signalling pathway may be involved in the action of different inhibitory hormones like PGE₂ on haematopoiesis.

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ACKNOWLEDGEMENTS. This study was supported by a research grant from the 'Deutsche Forschungsgemeinschaft' (De 404/2–1).

Received 12 December 1997;
accepted in revised form 25 February 1998



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