cAMP attenuates interleukin-1-stimulated macrophage colony-stimulating factor (M-CSF) expression

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Macrophage colony-stimulating factor (M-CSF) is a multi-functional cytokine attributed with key biological functions beyond the first discovered role in promoting proliferation of myeloid cell lineage. The human pancreatic cancer cell line MIA PaCa-2, from which the *M-CSF* gene was originally cloned, was used to study regulation of M-CSF expression. Expression of M-CSF was inducible by interleukin-1 α (IL-1 α), lipopolysaccharide (LPS) and PMA as demonstrated by a biological activity assay, Northern-blot analysis and reverse transcriptase (RT) PCR. Treatment of the cells with forskolin or dibutyryl-cAMP attenuated the expression of M-CSF induced by IL-1 α or LPS, but not by PMA. Electromobility shift assays showed that IL-1 α predominantly activated nuclear factor κ B (NF- κ B), while PMA preferentially activated activator protein-1 (AP-1). The activation

of NF- κ B, but not AP-1, could be attenuated by cAMP elevation. Relative RT-PCR demonstrated that the expression of a 1.6-kb M-CSF mRNA transcript was more effectively induced by IL-1 α than a 4.0-kb transcript. By and large the induced expression of both mRNA transcripts could be attenuated by cAMP. M-CSF promoter-driven luciferase reporter-gene assays revealed that cAMP elevation attenuated the IL-1-induced transcription activation of the M-CSF promoter, but it had no effect on PMA-induced transcription. Our findings suggest that cAMP regulates M-CSF gene expression at the transcriptional level and that its inhibitory effect involves NF- κ B signalling pathway.

Key words: activating protein-1, electromobility shift assay, NF- κ B, nuclear factor kappa-B, phorbol 12-myristate 13-acetate.

INTRODUCTION

Emerging experimental evidence implicates the essential roles of macrophage colony-stimulating factor (M-CSF) in several important biological processes other than proliferation and survival of monocytes/macrophages, such as placental development, bone formation, tooth eruption and enhancing tumoricidal activity [1]. Furthermore, recent reports also suggest that M-CSF is required in the pathogenesis of atherosclerosis [2] and development of osteoporosis [3,4]. While most studies have focused on the effects of M-CSF and the signal transduction after the engagement of the M-CSF receptor, a limited amount of information regarding regulation of *M-CSF* gene expression itself exists.

M-CSF is encoded by a single gene on the long arm of chromosome 1 [5], which comprises 10 exons spanning 21 kb [6]. Alternative splicing occurring within exon 6 primarily generates mRNA transcripts that derive soluble or membrane-bound forms of the M-CSF. The major species of mRNA transcripts present is a 4.0-kb product, which contains the full coding sequence and gives rise to a translated protein of 522 amino acids [7]. This form of M-CSF is synthesized as an integral membrane glycoprotein and it is intracellularly processed and subsequently becomes soluble growth factor. The 1.6-kb mRNA transcript, which lacks the sequence of exon 6, is minimally expressed. Its protein product is bound to the cell membrane more stably and can be cleaved and released to the extracellular fluid slowly [8]. Both forms of M-CSFs are biologically active. A distinctive function

of the membrane-bound form is believed to be providing a cell-surface biological activity for local cell–cell signalling [9].

Previous studies from this laboratory and others have shown that interleukin (IL)-1, tumour necrosis factor (TNF), phorbol ester, endotoxin, cGMP and calcium/calcium ionophores stimulate M-CSF production in human pancreatic cancer cell line MIA PaCa-2 cells, as well as the human lung fibroblast cell line CCL202 [14]. Contrarily, cAMP and cAMP-elevating agents such as forskolin, pertussis and cholera toxins and theophylline reduced the IL-1-induced M-CSF production as detected by a biological activity assay [14,15].

The 5'-flanking region (570 bp upstream) of the gene was reported to contain putative *cis*-acting sites for transcription factors activator protein 1 (AP-1), nuclear factor κ B (NF- κ B), early growth-response protein ('EGR')-1 and NF-IL6 (sometimes designated C/EBP- β , CCAAT-enhancer binding protein- β) [10]. The interaction between these regulatory transcription elements would be expected to contribute to *M-CSF* gene expression. A region containing the AP-1-binding site is required for the basal expression of murine *M-CSF* gene [11]. The expression of c-Fos and c-Jun was observed by Northern blotting before the expression of M-CSF in U937 cells stimulated with PMA [15]. Therefore, it appears that AP-1 is possibly one of the important regulatory elements for M-CSF expression.

The NF- κ B element is a ubiquitous transcription factor, which can activate a number of proinflammatory cytokines and other genes [20]. NF- κ B activation is a major signalling pathway stimulated by IL-1, one of the potent acute-phase-response

Abbreviations used: M-CSF, macrophage colony-stimulating factor; IL, interleukin; LPS, lipopolysaccharide; TNF, tumour necrosis factor; RT-PCR, reverse transcriptase PCR; DB-cAMP, dibutyryl-cAMP; EMSA, electromobility shift assay; NF- κ B, nuclear factor κ B; AP-1, activator protein-1; DMEM, Dulbecco's modified Eagle's medium; I κ B, inhibitory κ B.

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cytokines. A segment of the 5'-flanking region of *M-CSF* gene (positions -419 to -304) that includes the binding site for NF- κ B is required for TNF-induced *M-CSF* gene expression in HL-60 cells as well as for minimally modified low-density-lipoprotein-mediated M-CSF induction in mouse L-cells [12,13].

Activation of the protein kinase A-dependent signalling pathway by cAMP is an important pathway for regulating cytokine expression. Activation of protein kinase A down-regulates the expression of IL-2 and IL-2 receptor [16,17], as well as IL-3 and granulocyte/M-CSF [18] in human T-cells. Several agents that raise intracellular cAMP, such as 8-bromo-cAMP, 3-isobutyl-1-methylxanthine, cholera toxin and prostaglandin E₂, inhibit DNA synthesis in bone marrow-derived macrophages [19]. And we have also shown that cAMP negatively regulates M-CSF expression.

The present studies have examined the functional significance of transcription factors NF- κ B and AP-1, as well as their interactions with cAMP-dependent signalling during IL-1-, lipopolysaccharide- (LPS-) and PMA-induced activation of *M-CSF* gene expression in MIA PaCa-2 cells. Our results indicate that *M-CSF* gene can be induced via at least two distinct signalling pathways. IL-1 and LPS stimulation preferentially depended on the activation of transcription factor NF- κ B, which could be attenuated by intracellular cAMP elevation. On the other hand, PMA, which predominantly utilized transcription factor AP-1, was not affected by cAMP-dependent signalling. The promoter/reporter assays confirmed that cAMP elevation negatively affected the IL-1 signalling upstream to the *M-CSF* gene transcription.

MATERIALS AND METHODS

Materials

All standard tissue-culture reagents were obtained from Hyclone (Logan, UT, U.S.A.). Recombinant human IL-1 α [IL-1 α (117-271)] Ro24-5008 was a gift from Hoffmann La Roche (Nutley, NJ, U.S.A.). Bacto[®] Lipopolysaccharide (*Salmonella typhosa* LPS) was obtained from Sigma (St. Louis, MO, U.S.A.). [γ -³²P]ATP and [α -³²P]dCTP were purchased from Dupont NEN (Boston, MA, U.S.A.). The plasmids pGL2 and pSV- β -galactosidase were from Promega (Madison, WI, U.S.A.). FuGene-6 was supplied by Roche (Indianapolis, IN, U.S.A.). Unless otherwise stated all other biochemical reagents were purchased from Sigma.

Cell culture and treatment

The human pancreatic carcinoma cell line MIA PaCa-2 obtained from the A.T.C.C. was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 7.5% fetal calf serum and 2.5% horse serum. Subculture of cells is done by treatment with 0.05% trypsin/0.02% EDTA and resuspension in fresh DMEM with 10% fetal-calf serum. Cells were cultured at 37 °C in a humidified incubator under 6% CO₂. When 90% confluence was reached, cells were washed twice with PBS before switching to serum-free DMEM. In specified cultures, recombinant IL-1 α , PMA, the calcium ionophore A23187 or LPS were added to the cell cultures at final concentrations of 17 ng/ml, 20 ng/ml, 1 μ M and 100 ng/ml respectively. Cells were incubated further and harvested at specified times.

Assay for M-CSF activity

The soft-agar colony assay of bone marrow cells was carried out as described elsewhere [14]. Mouse bone marrow cells were

obtained from C57BL/6J mice from Jackson Laboratory (Bar Harbor, ME, U.S.A.). Colony counts were performed using a dissection microscope at day 5, and aggregates of over 50 cells were scored as colonies. A unit of M-CSF activity was defined as the amount needed to form one colony. For morphological analysis of the colonies, the entire soft-agar plates were mixed, dried and stained with haematoxylin (Sigma) according to the company's instructions.

Nuclear-protein extraction

Nuclear-protein extracts were prepared according to Dignam et al. [22]. After incubation under a specific treatment, (1.5–2) $\times 10^6$ cells were washed twice with PBS before being scraped into buffer A [10 mM Hepes (pH 7.9), 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol and 0.5 mM PMSF] and briefly centrifuged. Cell pellets were consequently resuspended in buffer A, kept on ice for 15 min, and 10% Nonidet P-40 was added. Then pellets were homogenized in a Dounce homogenizer and centrifuged at 10000 *g* for 10 s at 4 °C. Nuclear pellets were resuspended in extraction buffer [20 mM Hepes (pH 7.9), 0.55 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM PMSF and 10 mg/ml leupeptin], homogenized and centrifuged at 10000 *g* for 2 min at 4 °C. Samples in the supernatants were collected and stored at -70 °C until used. Concentrations of the nuclear-protein samples were determined by Bio-Rad protein assay according to manufacturer's protocol.

Electromobility shift assay (EMSA)

Synthetic oligonucleotides with the sequences corresponding to *cis*-acting elements of the M-CSF promoter were ³²P 5'-end-labelled with T4 polynucleotide kinase. Equal amounts (2–10 μ g) of nuclear extract from each of various cell treatments were incubated in 20 μ l of binding reaction [final concentrations: 40 mM KCl, 15 mM Hepes (pH 7.9), 1 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM PMSF, 5% glycerol and 1 μ g of poly-(dIdC)] for 10 min. Then, equal amounts of 60000 c.p.m. (approx. 1 ng of DNA) of radiolabelled oligonucleotide probe were added to the reaction mixture, and incubated further for an additional 25 min at room temperature. DNA-protein complexes were separated from unbound DNA probe by electrophoresis on native 5% polyacrylamide gels at 140 V in 45 mM Tris/borate (pH 8.0) containing 1 mM EDTA and then autoradiographed. Oligonucleotide probes (Biosynthesis, Lewisville, TX, U.S.A.) with the following sequences; NF- κ B, 5'-agttgaGGACTTCCagge-3' (positions -368 to -377 of the M-CSF promoter), and AP-1, 5'-cgcttgATGTGTCAgTccggaa-3' (positions -538 to -531 and -344 to -337 of the M-CSF promoter) were used. The upper-case letters are the consensus sequences for the NF- κ B and AP-1 binding sites respectively. The gel-supershift antibodies against p65 and c-Jun/AP-1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). The mutant NF- κ B and mutant AP-1 gel-shift oligonucleotides were also purchased from Santa Cruz Biotechnology.

RNA extraction and Northern-blot analysis

Total cellular RNA was isolated using TRIpure[™] reagent (Boehringer Mannheim) as per the manufacturer's protocol. Samples containing equal RNA (15–20 μ g) were fractionated in 1.2% agarose gels and transferred to nylon membranes. The membranes were then hybridized according to standard protocol [23] with either cloned M-CSF cDNA or with probes generated by PCR for M-CSF- or human 18 S rRNA-specific cDNA. The probes were radiolabelled with [α -³²P]dCTP by random priming

using Klenow fragment from Promega as per the manufacturer's direction. After hybridization, InstantImager[®] Electronic Autoradiography (Packard, Meriden, CT, U.S.A.) was used to detect and quantify hybridization signals.

Relative reverse transcriptase PCR (RT-PCR)

Total RNA (1 μ g) from cells was reverse-transcribed using Moloney murine leukaemia RT from Life Technologies (Gaithersburg, MD, U.S.A.) at 42 °C for 1 h. A 2- μ l aliquot of the diluted cDNA was amplified by PCR using recombinant *Taq* polymerase (Promega) according to the manufacturer's instructions. The M-CSF PCR primers were designed as described previously [21]: M-CSF-A (sense), 5'-CATGACAAGGCCTGCGTCCGA-3' (nt 489–409); M-CSF-B (antisense), 5'-AAGC-TCTGGCAGGTGCTCCTG-3' (nt 762–782); M-CSF-C (antisense), 5'-GCCGCCTCCACCTGTAGAACA-3' (nt 1547–1567). Human 18 S rRNA primers, 5'-ACGGCTACCACATCCAAG-3' (sense) and 5'-CGCCCCGCCGCTCCCAAGA-3' (antisense) [24], were used in the PCR reactions as loading controls. Amplicons corresponding to the full-length 4.0-kb (using M-CSF-A and M-CSF-B primers) and the exon-6-spliced 1.6-kb (using M-CSF-A and M-CSF-C primers) M-CSF mRNAs with molecular sizes of 395 and 286 bp, respectively, were electrophoresed on 2% agarose gel to determine their sizes. Signals were subsequently confirmed by Southern hybridization with radiolabelled M-CSF cDNA. The human 18 S PCR product was 200 bp in length.

Plasmid constructs, cell transfection and reporter-gene assay

A plasmid containing the 5' region of the human *M-CSF* gene promoter was kindly provided by Dr Hisashi Yamada (Jeikei University Medical School, Minato-ku, Tokyo, Japan). After digestion by *Sst*I and *Hind*III, a 570-bp fragment of the 5' M-CSF promoter was directionally subcloned upstream to the firefly luciferase gene in plasmid pGL2 (Promega) at the *Sac*I/*Hind*III site, deriving p570Luc, and was confirmed by sequencing. In all transfections, 1 μ g of pSV- β -galactosidase was used to monitor the transfection efficiency. MIA PaCa-2 cells were seeded at 2×10^5 cells/35-mm plate 24 h before transfection. Cells were washed twice with PBS and switched to serum-free DMEM 6 h before transfection. Transfection of plasmid DNA into cells was performed by using FuGene-6 according to manufacturer's protocol. Transfected cells were stimulated with IL-1 or PMA with or without forskolin 12 h after transfection, then incubated further for 12 h until harvested for luciferase assay (Promega) as per the manufacturer's protocol. β -Galactosidase assay was performed as described previously [25]. Induction of luciferase activity is reported as the mean from two separate experiments in performed in triplicate normalized by β -galactosidase activity.

Data analysis

Densitometry of the autoradiographed bands from Northern blots and EMSA were analysed quantitatively by the Alpha Imager[®] program version 3.23 (Alpha Innotech Corporation, San Leandro, CA, U.S.A.). All values are expressed as means $\pm$ S.E.M. compared with controls and among separate experiments. Two-tailed paired Student's *t* test was used to determine the significance of changes in densitometric measurements and colony-forming activities. A significant difference was taken as a *P* value of less than 0.05.

RESULTS

Stimulation of M-CSF production in MIA PaCa-2 cells

Cultured MIA PaCa-2 cells produced a low basal level of M-CSF without any stimulation, as apparent by 40 ± 1.79 colonies formed in the CSF-1 biological activity assay. By contrast, IL-1 (17 ng/ml) enhanced M-CSF secretion into the media by nearly 3-fold compared with untreated cells. LPS (100 ng/ml), PMA (10 ng/ml) and calcium ionophore (1 μ M) A23187 also stimulated M-CSF production in MIA PaCa-2 cells, by about 2.5-fold over controls (Figure 1A).

Effects of cAMP on M-CSF production

The role of intracellular cAMP elevation on M-CSF expression was examined by addition of either 1 μ M dibutyryl-cAMP (DB-cAMP; results not shown) or 5 μ M forskolin to the cultured MIA PaCa-2 cells stimulated by IL-1, LPS or PMA. Either DB-cAMP or forskolin treatment alone had no effect on basal M-CSF production. Intracellular cAMP elevation by either forskolin or DB-cAMP significantly attenuated both IL-1-induced and LPS-induced M-CSF production from 51.3 ± 2.12 to 35 ± 1.39 ($P = 0.006$) and from 46.3 ± 1.44 to 34.6 ± 1.45 ($P = 0.01$) respectively (Figure 1B), while there was no effect on PMA-induced M-CSF production ($P = 0.256$). This result suggests

Figure 1 Stimulation of M-CSF production and effect of intracellular cAMP elevation

(A) MIA PaCa-2 cells were cultured with effectors for 48 h at the final concentrations of IL-1 = 17 ng/ml, LPS = 100 ng/ml, PMA = 10 ng/ml and calcium ionophore A23187 = 1 μ M. The conditioned media were harvested and assayed for biological activity as detailed in the text. (B) Cultured MIA PaCa-2 cells were treated the same way in the presence (hatched and dotted bars) or absence (unfilled and filled bars) of 5 μ M forskolin. Data are means $\pm$ S.E.M. of colony numbers for triplicate determinants from one representative of three similar experiments. * and ** depict significant differences ($P < 0.05$) between samples treated with effector only versus samples with forskolin added.

Figure 2 Effect of forskolin on stimulated M-CSF mRNA expression

Total RNA (20 μ g/lane) from MIA PaCa-2 cells treated with IL-1, LPS or PMA in the presence or absence of 5 μ M forskolin was hybridized with radiolabelled M-CSF cDNA fragment in Northern analyses. RNA loading was determined by quantification of human 18S rRNA (h18S). The blot is representative of three similar independent experiments.

that PMA utilizes a different signalling pathway to IL-1 and LPS to induce M-CSF expression.

Northern-blot analyses revealed that IL-1 induced the mRNA expression of M-CSF in a time-dependent manner. The peak of induction occurred between 4 and 6 h after IL-1 stimulation (results not shown). Intracellular cAMP elevation by forskolin significantly lowered both IL-1- and LPS-induced expression of M-CSF at 6 h after treatment. On the other hand, no attenuation of PMA-induced mRNA induction was observed (Figure 2). The attenuation of M-CSF biological activity, in conditioned media, by forskolin treatment is consistent with a decrease of intracellular mRNA accumulation, suggesting that cAMP elevation affects the expression of M-CSF at the transcriptional level.

Relative RT-PCR was also performed to detect any changes in the minor mRNA transcript of M-CSF (1.6-kb mRNA) concomitant with those of the major mRNA transcript (the 4.0-kb mRNA), because 1.6-kb transcripts are rarely detected by Northern blotting. Total RNA from MIA PaCa-2 cells were cultured with or without IL-1 and forskolin for 4 h. IL-1-induced expression of both 4.0- and 1.6-kb M-CSF mRNA transcripts increased significantly over the untreated control (Figures 3A and 3B). Forskolin treatment alone did not cause any apparent change of the mRNA over the control. Inducibility of the 1.6-kb mRNA transcripts by IL-1 was approx. 11-fold over the control, while that of 4.0-kb transcripts were induced at about a 3-fold higher level. Expression of both 1.6- and 4.0-kb transcripts was significantly attenuated by forskolin ($P = 0.003$ and 0.001 , respectively). Similar to the degree of inducibility, forskolin attenuated the IL-1-induced 1.6-kb transcripts to a greater extent (approx. 50%) than the 4.0-kb transcripts (approx. 30%; Figure 3C).

Activation of NF- κ B

IL-1 α (17 ng/ml), 10 ng/ml PMA or 100 ng/ml LPS was added to the cultured MIA PaCa-2 cells for 60 min and a nuclear-protein extract from each treatment was prepared. The doses of stimuli used throughout the study were the lowest concentrations that yielded maximum M-CSF biological activity, as determined previously by a mouse bone marrow cell colony-forming assay. Next, equal amounts of protein (10 μ g) from each extract were assayed for NF- κ B activity by EMSA. NF- κ B binding complex appeared as a sharp single band on the autoradiogram (Figure 4, top panel, lanes 5 and 7). In these experiments, NF- κ B was activated strongly by IL-1 α and to the lesser degree by LPS.

Figure 3 Expression of M-CSF mRNA transcripts and effect of forskolin

Relative RT-PCR detecting induction of the mRNA transcripts of (A) membrane-bound (1.6-kb) and (B) soluble (4.0-kb) M-CSF in MIA PaCa-2 cells stimulated by 17 ng/ml IL-1 for 6 h (I), either with (F) or without 5 μ M forskolin. A densitometry measurement is shown in (C). * Represents a significant difference ($P < 0.05$) between cells treated with IL-1 only versus ones with 5 μ M forskolin for both 4.0- and 1.6-kb M-CSF mRNA transcripts. C, control; h18S, human 18S rRNA.

PMA treatment either with or without forskolin weakly activated NF- κ B binding. No significant activation of NF- κ B was observed when the cells were treated with forskolin alone (Figure 4, top panel, lanes 6 and 7). Elevation of intracellular cAMP levels, by pretreatment of the MIA PaCa-2 cells with 5 μ M forskolin 3 h prior to the stimulation by IL-1 or LPS treatment, markedly attenuated NF- κ B activation (Figure 4, top panel, lanes 4 and 6). When a mutant NF- κ B probe that differed from the wild-type sequence by 1 nt was used with the IL-1-treated extract to confirm the band identity, no binding was observed (Figure 4, top panel, lane 10). This indicated that the binding complex was sequence-specific. A polyclonal antibody to the NF- κ B p65 subunit, which recognizes epitope corresponding to amino acids 3–19 within N-terminal domain, was used in a supershift experiment. The antibody further retarded the protein–DNA complex and the band appeared to be supershifted from the original position (Figure 4, bottom panel). By contrast, control generic IgG had no effect on the protein–DNA complex. This result further confirmed the identity of the NF- κ B binding complex formed.

Activation of AP-1

Similar nuclear-protein extracts were used in EMSA with the oligonucleotides containing the consensus-binding sequence for AP-1. AP-1 activation was also detected as a single band on the corresponding autoradiogram (Figure 5A). PMA and LPS strongly increased the binding of AP-1 (from an integrated absorbance area of 1614 in the control to 4584 and 4288, respectively; Figure 5A, lanes 4 and 6). IL-1 elicited an apparently much weaker AP-1 activation (absorbance, 2599; Figure 5A, lane 8). On the other hand, no AP-1 binding appeared when the mutant radiolabelled AP-1 probe was used with the PMA-treated nuclear extracts (Figure 5A, lane 10). Forskolin did not significantly change AP-1 binding by itself (absorbance, 1586) compared with the control. Furthermore, it did not have any apparent effect on PMA-, LPS- or IL-1-induced AP-1 binding

17 ng/ml IL-1	-	-	-	-	+	+	-	-
5 μ M Forskolin	-	+	+	-	+	-	+	-
100 ng/ml LPS	-	-	+	+	-	-	-	-
10 ng/ml PMA	-	-	-	-	-	-	+	+

Figure 4 NF- κ B activation complex in stimulated MIA PaCa-2 cells

(Top panel) MIA PaCa-2 cells were harvested and extracted for nuclear proteins 1 h after treatment by IL-1, LPS or PMA. Cells were pre-incubated with 5 μ M forskolin or 1 μ M DB-cAMP for 3.5 h before the effectors were added (where specified). NF- κ B oligonucleotide probes were incubated with 10 μ g of nuclear extract/lane and resolved on 5% polyacrylamide gel. Mutant NF- κ B probe was used with IL-1-treated extract in lane 10 (reading left to right); lane 1 was a wild-type probe without nuclear extract. (B) Anti-p65 subunit antibody (1 μ g; 5 μ l) or 1 μ g of preimmune IgG was added to the binding reaction containing 10 μ g of IL-1-treated nuclear extract and wild-type NF- κ B oligonucleotides, further incubated for 15 min, and resolved on 5% polyacrylamide gel.

(absorbances 4474, 4239 and 2546, respectively; Figure 5A, lanes 5, 7 and 9). Here, a supershift experiment was performed with a polyclonal antibody to c-Jun/AP-1, which was designed to tether to residues 247–263 within the DNA-binding domain of AP-1. The supershift band was clearly apparent, but the intensity of the retarded band at the original position was reduced only minimally (Figure 5B).

The binding between transcription factors and the oligonucleotides in the protein–DNA complexes was sequence-specific for both NF- κ B and AP-1. Mutant probes, which differ from the consensus oligonucleotides by one amino acid, could not elicit binding. Thus the identity of the protein–DNA complexes was proven. The supershift caused by the specific antibodies to NF- κ B and c-Jun/AP-1 further confirmed the involvement of these transcription-factor proteins in the formation of these protein–

A. 17 ng/ml IL-1	-	-	-	-	-	-	+	+
5 μ M Forskolin	-	+	-	+	-	+	-	+
100 ng/ml LPS	-	-	+	+	-	-	-	-
10 ng/ml PMA	-	-	-	-	+	+	-	-

Figure 5 AP-1 activation complex in stimulated MIA PaCa-2 cells

(A) Equal amounts of 32 P-labelled AP-1 oligonucleotide probe were incubated with 6 μ g/lane of nuclear protein from MIA PaCa-2 cells stimulated with the indicated treatment. Mutant 32 P-labelled AP-1 probes incubated with PMA-treated extract was in lane 10 (reading left to right); lane 1 was probe only. (B) Identity of AP-1 binding was also confirmed by supershift experiment using anti-c-Jun/AP-1 antibody (1 μ g, 5 μ l) or preimmune IgG (1 μ g) to incubate with PMA-treated nuclear extract and wild-type AP-1 probes at 4 $^{\circ}$ C for 12 h before resolving on 5% polyacrylamide gel.

DNA complexes. This protein–DNA complex formation reflects the first step in the gene transcription that transcription factors engage in the pre-initiation complex at the corresponding *cis*-acting binding sites. The anti-c-Jun/AP-1 antibody formed the distinct supershift band without significant weakening of the original AP-1 binding (Figure 4, bottom panel). This was probably due to insufficient quantity of the antibody used compared with the amount of nuclear-protein extract.

Transcription activity of *M-CSF* gene promoter

To determine the IL-1 and PMA inducibility of the *M-CSF* gene promoter at the level of gene transcription, we examined its functional potential on a reporter gene. MIA PaCa-2 cells were transfected with the plasmid containing the firefly luciferase cDNA under control of full-length M-CSF promoter (p570Luc). We detected an approx. 10-fold increase in relative luciferase activity in the transiently transfected MIA PaCa-2 cells treated

Figure 6 Effect of forskolin on transactivation of (A) IL-1-induced and (B) PMA-induced M-CSF promoter-driven luciferase activity

MIA PaCa-2 cells were transiently transfected for 12 h prior to switching to serum-free media containing specified treatments and incubated further for 12 h. Cells were then harvested for reporter-gene assays. (A) Transfected cells were treated with 5 μ M forskolin, 17 ng/ml IL-1 or a combination of both. (B) Treatments were 5 μ M forskolin, 10 ng/ml PMA or a combination. The data are expressed as the activity of the luciferase relative to β -galactosidase expressed as the means $\pm$ S.E.M. of two experiments, each of which was performed in triplicate. The asterisk shows a significant difference ($P < 0.05$) between transfected cells treated with IL-1 only versus ones co-treated with 5 μ M forskolin.

with 17 ng/ml IL-1 compared with untreated cells after 12 h (Figure 6A). Similarly, transfected cells treated with 20 ng/ml PMA yielded about a 6-fold increase in luciferase activity (Figure 6B). A control reporter plasmid containing luciferase gene driven by cytomegalovirus promoter yielded no significantly different luciferase activities between untreated and IL-1- or PMA-treated transfections, suggesting that induction of luciferase activity requires specific elements in the human *M-CSF* gene promoter (results not shown). Forskolin alone did not cause significant change in luciferase activity compared with the untreated cells. Co-treatment with forskolin distinctively attenuated IL-1-induced relative luciferase activity from 9.65 ± 0.804 to 6.67 ± 0.415 ($P = 0.011$). By contrast, forskolin had no significant effect on luciferase activity induced by PMA in transfected MIA PaCa-2 cells (from 5.73 ± 0.972 to 5.40 ± 0.289 , $P = 0.374$).

DISCUSSION

In addition to DB-cAMP, forskolin and theophylline were reported earlier to increase intracellular cAMP levels and suppress IL-1-induced M-CSF production [14]. In this study, our results show that intracellular cAMP elevation attenuated IL-1-

induced and LPS-induced M-CSF production by approx. 50 %, while the PMA-induced M-CSF production in MIA PaCa-2 cells was unaffected by forskolin. The changes in mRNA message detected by Northern hybridization correlated well with the changes in biological activities for both stimulatory and inhibitory stimuli. There is also evidence from previous reports in various cells indicating that expression of M-CSF is regulated primarily at the level of gene transcription [25,26]. Two conjectures may be drawn, first that *M-CSF* gene expression is regulated at the transcriptional level and, secondly that cAMP elevation adversely affects the *M-CSF* gene expression at or upstream of the transcriptional level. The results from the promoter/reporter-gene assays strongly support this. Recent reports demonstrated that LPS receptor or toll-like receptor (TLR 2 and 4) conferred LPS responsiveness through activation of NF- κ B via the signalling molecules shared by IL-1 signalling [27,28]. Thus, it is reasonable to expect that both IL-1- and LPS-induced M-CSF expression were affected by cAMP elevation in a similar manner.

Differential effects of intracellular cAMP elevation on these particular different signalling pathways, which regulate the same target genes, are not unprecedented. Activation of the cAMP-dependent signalling pathway by DB-cAMP or prostaglandin E2 resulted in the down-regulation of IL-4 in concanavalin A- or anti-CD3/anti-CD28-activated human T lymphocytes. By contrast, neither DB-cAMP nor prostaglandin E2 affected IL-4 expression in PMA/A23187-activated T cells [29]. Other inducible genes for which expression can be attenuated by cAMP elevation include IL-2 and nitric oxide synthase [30,31]. These genes are commonly regulated by NF- κ B, so it is probable that cAMP negatively affects one of the signalling components in the NF- κ B activation pathway. A critical step in the signal-induced activation of NF- κ B is the site-specific phosphorylation of its inhibitor, inhibitory κ B (I κ B). This commits the latter for degradation by the ubiquitin-proteasome pathway [22]. In EL-4 cells, the suppressive effect of cAMP on IL-2 expression involved inhibition of the binding of p50/p65 heterodimers to the NF- κ B site as well as the alteration of the complexes bound to the nuclear factor of activated T cells ('NF-AT') site [30]. The mechanism that forskolin employs to down-regulate LPS-stimulated inducible nitric oxide synthase induction was attributed to its ability to prevent the degradation of I κ B α and also to induce I κ B α expression [31]. Recently both the NF- κ B-inducing kinase as well as its downstream enzyme complex inhibitory κ B kinase were identified as upstream signalling molecules of I κ B [32]. This provides the possibility that any of these signalling components can be the target, upon which cAMP exerts its inhibitory effect.

NF- κ B and AP-1 are inducible transcription factors critical for the expression of many genes involved in the inflammatory response, including adhesion molecules and cytokines. The enhancer region of the *M-CSF* gene contains two NF- κ B-binding sites as well as two AP-1-binding sites. IL-1 is generally regarded as an activator of both NF- κ B and AP-1. In MIA PaCa-2 cells, IL-1 α strongly activated NF- κ B, but was not a particularly strong stimulator of AP-1. A comparable circumstance was found in TNF α , a cytokine with a similar signalling mechanism to IL-1, which induced both AP-1 and NF- κ B in A549 epithelial cells, but only NF- κ B in HMEC-1 [32]. PMA greatly activated AP-1, while it only modestly evoked NF- κ B activation in MIA PaCa-2 cells. PMA acts as a diacylglycerol analogue and thus induces protein kinase C as well as phospholipase C activation. This suggests that *M-CSF* gene induction can be achieved through at least two different signal-transduction pathways. The preferential activation of one transcription factor over the other

by one specific stimulus leads to the question as to whether activation of both NF- κ B and AP-1 simultaneously are required to induce *M-CSF* gene expression. A currently evolving model of transcriptional activation comes from the studies of the interferon- γ gene expression. This illustrates that an assortment of transcription factors in the enhancer region co-operatively induces transcription activation to varying degrees and all of the *cis*-acting elements are required in a specific architectural context to exert maximum transcription activation [33,34]. Although IL-1 did not increase AP-1 binding over the unstimulated cells in EMSA, a noticeably high basal AP-1 activation could sufficiently co-operate with NF- κ B or another transcription factor to enhance *M-CSF* gene transcription. On the other hand, the observation that IL-1 α and PMA preferentially activate one transcription factor but not the other may reflect the fact that each of them does not evoke a maximum *M-CSF* gene induction. Nevertheless, all three stimuli induced the expression of M-CSF in MIA PaCa-2 cells to a comparable degree, as observed by Northern analysis. However, NF- κ B and AP-1 are not the only known inducible transcription factors of which binding sites are present in the *M-CSF* enhancer region. Sp-1 binding has also been shown to be activated by acute-phase cytokines and in turn increases M-CSF expression [4]. The expression of the membrane-integrated form of M-CSF (1.6-kb mRNA) seemed to be more readily induced by IL-1 α than that of the major soluble 4.0-kb form. Interferon- γ stimulation also resulted in much higher inducibility in the expression of 1.6-kb mRNA than that of the 4.0-kb mRNA [21]. These findings fit the hypothesis that stromal cells may efficiently produce membrane-bound M-CSF when they are stimulated by acute-phase cytokines in the inflammatory state. This in turn may contribute to increases in the local concentration of M-CSF at the inflammatory sites. Although the implication of the expression of minimally expressed 1.6-kb mRNA is still not fully understood, our results suggest that both forms of mRNAs are attenuated by cAMP elevation. This further supports the idea that cAMP elevation affects M-CSF regulation at or upstream of the transcriptional level.

The M-CSF promoter/luciferase reporter-gene assays demonstrated that both IL-1 and PMA were able to induce transcription activation of the full-length M-CSF promoter to a distinctly different degree. The finding that cAMP elevation negatively affected only IL-1-induced- but not AP-1-induced-transcription activation of the same promoter sequence, in conjunction with the finding that IL-1 preferentially activated NF- κ B, narrows the target of cAMP attenuation of M-CSF expression to an NF- κ B-dependent signalling pathway upstream of gene transcription.

Both NF- κ B and AP-1 have their distinctive roles in *M-CSF* gene induction. Our findings show that attenuation of induced *M-CSF* gene expression by cAMP elevation involves NF- κ B-dependent signalling and suggest that it affects transcription regulation. IL-1 α not only up-regulated M-CSF gene expression, but also affected the alternative splicing of the gene.

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