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Regulation of gene expression of macrophage-colony stimulating factor in human fibroblasts by the acute phase response mediators interleukin (IL)-1β, tumor necrosis factor-α and IL-6

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Fibroblasts constitute a major element of the bone marrow stroma. They play a pivotal role in blood cell development by providing the scaffolding required for cellular organization and tissue cohesion and by producing soluble molecules including colony stimulating factors (CSFs) and various interleukins regulating hematopoiesis. Our data demonstrate that the acute phase response mediators interleukin (IL)-1 β, tumor necrosis factor (TNF)-α and IL-6 which are abundantly produced by activated monocytes, enhance levels of macrophage-colony stimulating factor (M-CSF) in fibroblasts by both transcriptional and post-transcriptional mechanisms. The action of these proteins to induce M-CSF transcript levels was dependent on synthesis of new proteins and was not mediated by protein kinase C (PKC) stimulation as depletion of cellular PKC pools by prolonged exposure of fibroblasts to phorbolester TPA did not prevent factor induced synthesis of M-CSF transcripts. However, blockade of PKC by the isoquinoline sulfonamide derivative H7 and thus inhibition of phosphorylation was associated with augmentation of the fibroblasts response to TNF-α and IL-6.

Macrophage-colony stimulating factor; Gene expression; Regulation; Protein kinase C

1. INTRODUCTION

The dynamic nature of the hematopoietic system suggests a flexible and complex regulation. In recent years much has been learned regarding soluble factors that govern hematopoietic blood cell development both in vitro and in vivo (see [1,2] for review). The formation of monocytes/macrophages has been shown to be controlled by a cytokine termed macrophage-colony stimulating factor (M-CSF), which in addition to its activity on macrophage progenitor cells stimulates the function of mature mononuclear phagocytes [3–5]. M-CSF also seems to play a role in other organ systems as reflected by its expression in placental tissue [6] and its inhibitory activity on bone formation [7]. Cellular sources of M-CSF include mononuclear phagocytes [8–10], polymorphonuclear phagocytes [11], fibroblasts [12–14], endothelial cells [15,16], epithelial cells of the thymus [17], B-lymphocytes [18] and placental tissue [6]. Although detectable levels of M-CSF in the circulation suggest its constitutive synthesis [19], secretion of M-CSF in culture is low or even undetectable unless producer cells are activated. Acute phase response mediators such as IL-1 β and TNF-α have been shown to enhance M-CSF expression in mononuclear phagocytes [9] and mesenchymal cells [12,13,15,16], suggesting that M-CSF production can be augmented on demand, and that acute phase mediators are involved in this process. Enhancement of steady state M-CSF production might be necessary in situations requiring an increased number of functionally competent macrophages, e.g. in inflammatory responses. In the studies to be reported below we show that IL-1 β, TNF-α and IL-6 elevate levels of M-CSF mRNA in fibroblasts and we analyze mechanisms involved in this process.

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Abbreviations: ANAE, α-naphtylacetate-esterase; CHX, cycloheximide; CM, conditioned medium; CSFs, colony stimulating factors; FCS, fetal serum calf; H7, 1-(isoquinolinylsulfonyl)-2 methylpiperazine; IL, interleukin; M-CSF, macrophage-colony stimulating factor; PAGE, polyacrylamide gel electrophoresis; PKC, protein kinase C; RP-HPLC, reversed-phase high-performance liquid chromatography; SDS, sodium dodecyl sulfate; SSC, standard sodium citrate (1 × SSC = 0.15 M NaCl, 0.015 M sodium tricitrate, pH 7.0); TNF, tumor necrosis factor; TPA, 12-O-tetradecanoylphorbol-13-acetate

2. MATERIALS AND METHODS

2.1. *Fibroblast preparation and culture*

Normal human embryonic lung fibroblasts (strain FH109) were isolated by proteolytic dispersion [20]. Cells were cultured and passaged by methods detailed elsewhere [20]. In all experiments, fibroblasts from passage 7–12 were used. Cultures were performed in α medium supplemented with 10% low endotoxin FCS (Boehringer, Mannheim, Germany), 100 μg/ml penicillin, 100 μg/ml streptomycin, 1 mM L-glutamine, 1 mM sodium pyruvate (Gibco Laboratories,

Heidelberg, Germany) in a humidified atmosphere containing 7% CO_2 in air at 37°C. The culture period was 2–72 h.

Conditioned medium (CM) from cultures of fibroblasts that were incubated with IL-1 β (100 U/ml), TNF-α (10^3 U/ml) or IL-6 (10^3 U/ml) was collected, filtered and stored at −20°C until use. In some experiments cultures received H7 (30 μM), TPA (8–24 nM), actinomycin-D (5 μg/ml), or cycloheximide (20 μg/ml).

2.2. *Reagents*

Recombinant (*Escherichia coli*-derived) human (rh) TNF-α (specific activity (SA) of 5×10^7 U/mg of protein) was kindly provided by Genentech, San Francisco, CA, through Dr G.R. Adolf, Ernst Boehringer Institute for Drug Research, Vienna, Austria. Recombinant human IL-1 β was kindly provided by Dr. D. Krumwieh, Behringwerke AG, Marburg, Germany). The SA was 10^9 U/mg. Recombinant human IL-6 was a kind gift of Dr. T. Hirano, Division of Immunology, Osaka University, Osaka, Japan. The SA was 5×10^5 U/mg of protein. Purity of TNF-α, IL-1 β and IL-6 was >99% by SDS-PAGE and RP-HPLC. Endotoxin content of all cytokine preparation investigated was < 50 pg/mg of protein as assessed by the limulus amoebocyte assay. A full length cDNA probe for human M-CSF (1 kb *BglI–PstI* fragment cloned into pUC 18) was kindly provided by Dr. P. Ralph, Cetus Corporation, Emeryville, CA, USA. The 2.0 kb *PstI* fragment of the chicken β-actin gene was derived from the pAI-plasmid (kindly provided by Dr. J. Ramadori, Dept. of Medicine, University of Mainz, Mainz, Germany). The probes were ^{32}P-labeled by random priming [21]. The specific activity was $4–8 \times 10^8$ cpm/μg. 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine (H7), 12-*O*-tetradecanoylphorbol-13-acetate (TPA), cycloheximide (CHX), 2-mercaptoethanol, actinomycin-D, trypsin, and EDTA were purchased from Sigma Chemicals, Munich, Germany.

2.3. *M-CSF bioassay*

M-CSF protein present in fibroblast CM was assayed by a double layer agar murine (endotoxin resistant C3H/HeJ mice) colony assay as described [10]. In brief, underlayers (0.5 ml) were composed of 0.5% agar (Agar Noble, Difco Laboratories, Detroit, MI, USA) in Iscoves modified Dulbeccos minimum essential medium (IMDM, Sigma) supplemented with 20% FCS, L-glutamine, penicillin/streptomycin (as for fibroblast cultures), and 10^{-5} M 2-mercaptoethanol. Fibroblast CM to be assayed for M-CSF biologic activity was added to the underlayers at 10% vol/vol final concentration. Control cultures received medium alone or rh M-CSF (750 U/ml). Overlayers (0.5 ml) were composed of 0.3% agar in the same medium and contained 5×10^4/ml bone marrow cells. Colonies derived from macrophage-colony forming units (M-CFU) were enumerated on day 7 after fixation and staining of whole agar cultures with α-naphtylacetate-esterase (ANAE).

2.4. *RNA extraction, Northern blot, and transcriptional run-on assay*

Total cellular RNA was isolated by lysing FH 10^9 cells in guanidinium isothiocyanate followed by recovery of RNA by centrifugation through cesium chloride [22]. After denaturation at 60°C, RNA was electrophoresed in an agarose formaldehyde gel (1.2%) and transferred to synthetic membranes (Schleicher and Schuell, Dassel, Germany). Filters were hybridized with labeled probe for 12–24 h at 42°C in 50% formamide, 2 × SSC, 5 × Denhardts, 0.1% SDS, 10% dextran sulfate and 100 μg/ml salmon sperm DNA. Filters were washed to a stringency of 0.1% SSC, 65°C for 12 min and used to expose Kodak Xomat-ray films with intensifying screens. To exclude incomplete RNA transfer in single lanes, all filters were reprobed with β-actin cDNA. Alterations in levels of M-CSF transcripts were quantitated by laser densitometry using multiple exposures of the blot and the ratio of M-CSF/β-actin transcripts in unstimulated cultures was assigned to be the baseline levels and were assumed to have 100% activity.

This ratio was compared to the ratio of experimental cultures. The fold change of M-CSF mRNA from base line levels was calculated by multiplication of the ratio of density of M-CSF/β-actin transcripts by the reciprocal of the ratio of base line levels. Nuclear run-on transcription assays were performed as previously described [20]. Briefly, nascent RNA chains were allowed to elongate in the presence of [^{32}P]uridine triphosphate. The ^{32}P-labeled nuclear RNA was purified by DNase and proteinase K digestion, phenol/chloroform extraction, and ethanol precipitation. Equivalent amounts of TCA-precipitable ^{32}P-labeled RNA were hybridized to vector DNA, β-actin and M-CSF probes immobilized on nitrocellulose filters. The ^{32}P-labeled RNA bound to the filters was visualized by autoradiography at −70°C by using intensifying screens.

2.5. *Protein kinase C assay*

Fibroblasts were suspended in serum-free RPMI 1640 medium and incubated in the presence of TPA (24 nM) at 37°C. 10^7 cells were then collected and pelleted in a microcentrifuge. The cell pellets were suspended in 100 μl distilled water, homogenized by passage through a 18-gauge needle and immediately reconstituted in a buffer containing 20 mM Tris-HCl, 100 μg/ml aprotinin, 0.25 mM leupeptin, 1 mM phenylmethyl-sulfonylfluoride, pH 7.5 (referred to as buffer A). The cytosolic fraction was obtained after centrifugation and the membrane fraction was solubilized in buffer A containing 1% Triton X-100 for 15 min at 4°C. Protein kinase C was separated by DEAE-cellulose column chromatography and elution with 80 mM NaCl. Aliquots of DEAE-purified protein kinase C were assayed for cytosolic and membrane activity in the presence of 10 mm $MgCl_2$, 1 mM $CaCl_2$, ca 10^6 cpm [γ-^{32}P]ATP (335 cpm/mmol; Amersham, Braunschweig, Germany) and 20 μg of histone H1, with or without 8 μg/ml phosphatidylserine. After incubation at 30°C for 10 min, protein kinase C activity was determined by subtracting the amount of ^{32}P incorporation into histone H1.

3. RESULTS

Passage 7–12 embryonic lung fibroblasts express M-CSF transcripts at low levels. However, 8 h following induction with the acute phase response cytokines TNF-α, IL-1 β, or IL-6, M-CSF transcripts are easily detectable in 10–20 μg of total cytoplasmic fibroblast RNA (Fig. 1A). Fibroblasts failed to release M-CSF protein into their culture supernatants unless culture was performed in the presence of acute phase response cytokines (Fig. 1B). An increase in transcript levels detected by Northern blot analysis can be due to an increase in the transcriptional rate of the respective gene, stabilization of previously transcribed mRNAs, or a combination of both mechanisms. As indicated in Fig. 2, TNF-α, IL-1 β, and IL-6 increased M-CSF mRNA stability. To examine post-transcriptional regulation of M-CSF expression, fibroblasts were cultured first in medium only or with acute phase response cytokines and then with actinomycin D. Cells were harvested at different time points (0–360 min) after adding actinomycin D and investigated for accumulation of M-CSF and β-actin mRNA. Half-life ($t_{1/2}$) of M-CSF mRNA in untreated cells was < 80 min. After stimulation with IL-1 β stability of M-CSF mRNA increased ($t_{1/2}$ = 360 min). Similarly to IL-1 β, treatment of fibroblasts with both IL-6 and TNF-α increased M-CSF mRNA half-life to 120 min and 290 min, respectively. In order to determine whether the rate of M-CSF transcription increases following fibroblast stimulation, nuclear transcription run-on

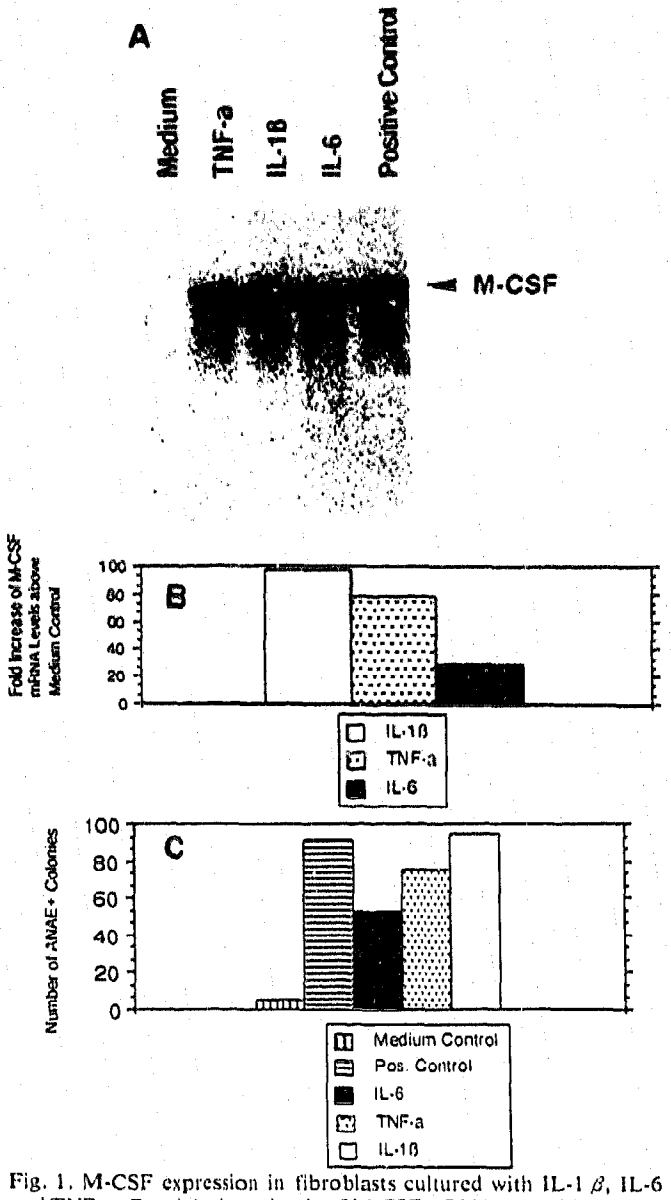

Fig. 1. M-CSF expression in fibroblasts cultured with IL-1 β, IL-6 and TNF-α. Panel A shows levels of M-CSF mRNA. Fibroblasts were exposed for 8 h to either medium only, rh IL-1 β (100 U/ml), rh IL-6 (10^3 U/ml) or rh TNF-α (10^3 U/ml). Cytoplasmic RNA (20 μg/lane) was prepared and analyzed by formaldehyde-agarose gel electrophoresis, transferred to synthetic membranes and sequentially hybridized with M-CSF cDNA and β-actin cDNA. The 4.0-kb hybridizing band is consistent with mRNA coding for M-CSF. β-actin controls for RNA loading in single lanes (not shown). The positive control represents mRNA derived from TPA-activated (12 nM, 8 h) peripheral blood monocytes hybridized with the M-CSF-specific cDNA. Panel B represents densitometric graphs (mean of two independent experiments with SD not exceeding 5%) of the same experiments as shown in panel A in which the data are normalized to actin mRNA levels. Panel C shows levels of M-CSF protein present in fibroblast cultures (72 h) that were performed in the presence of IL-1 β, IL-6 and TNF-α (concentrations as above). M-CSF biologic activity was measured by using the murine bone marrow CFU-M assay (ANAE$^+$ colonies) as described in section 2. Control cultures were performed with medium or rh M-CSF (750 U/ml). The experiment shown represents one of three independent experiments done with monocyte preparations of various donors. All experiments gave similar results with SD not exceeding 7.5%.

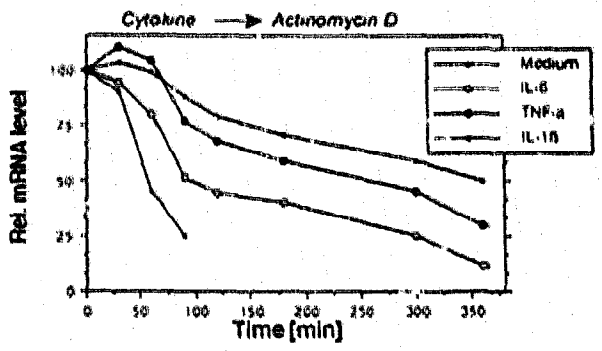

Fig. 2. Stability of M-CSF mRNA in fibroblasts cultured with IL-1 β, IL-6 and TNF-α. Fibroblasts were cultured with cytokines (concentrations as indicated in the legend to Fig. 1) or cultured with medium only for 8 h, and then actinomycin D (5 μg/ml) was added for 0, 30, 60, 90, 120, 240, 300 and 360 min. Cytoplasmic RNA was extracted and analyzed by Northern blotting (30 μg RNA per lane in untreated cells; 20 μg RNA per lane in treated cells). Intensity of hybridization was quantitated by laser densitometry. Untreated cells of each experiment were assumed to have 100% activity. In all experiments the data are normalized to the actin signal.

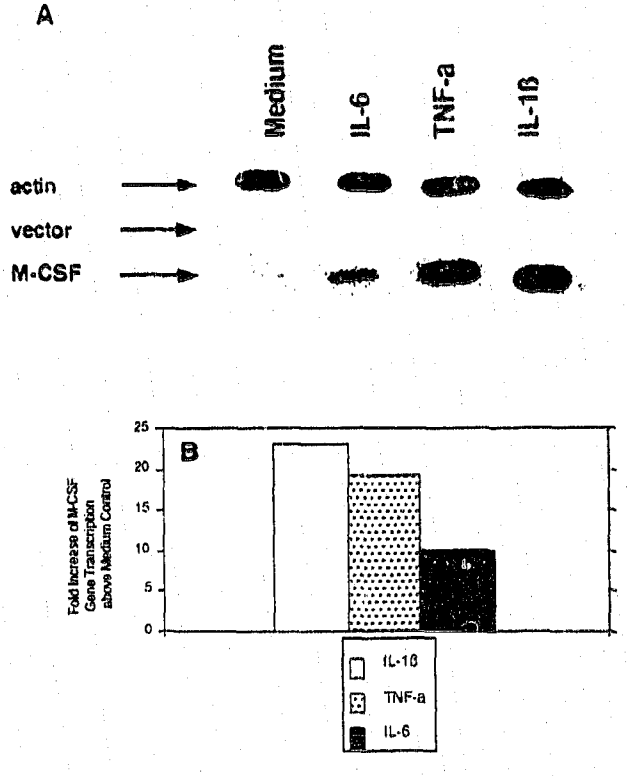

Fig. 3. Panel A: nuclear run-on assay analysis of the rate of ongoing transcription of M-CSF and β-actin in fibroblasts cultured in the presence or absence (medium only) of IL-6, TNF-α and IL-1 β (concentrations as indicated in the legend to Fig. 1). Nuclei were isolated from untreated cultures and 3 h following stimulation by cytokines. The in vitro ^{32}P-labeled RNA was hybridized to pUC vector DNA (vector) and to plasmids containing the human M-CSF and β-actin cDNA inserts. Panel B represents densitometric graphs (mean of two independent experiments with SD not exceeding 6%) of the same experiments as shown in panel A in which the data are normalized to actin transcription levels.

assays were performed at 3 h following exposure to IL-1 β, IL-6 and TNF-α. Concurrent samples were analyzed by Northern blot to confirm that total transcript levels increased at the time the transcription assay was performed (data not shown). As indicated in Fig. 3, stimulation of fibroblasts with all cytokines investigated increased the rate of transcription of M-CSF, with IL-1 β stimulating M-CSF transcription 23-fold, TNF-α 19-fold, and IL-6 10-fold by laser densitometry of autoradiographs. To analyze whether induction of M-CSF transcripts by acute phase response cytokines requires new protein synthesis, the effect of inhibition of protein synthesis on cytokine-induced M-CSF mRNA accumulation in fibroblasts was examined using CHX. Interestingly, CHX induced M-CSF transcripts within 30 min (Fig. 4), while in kinetic studies not shown here, enhancement of M-CSF transcripts by acute phase response cytokines was not detected until 90 min, suggesting that the M-CSF gene is controlled by a short-lived repressor molecule. However, when cells were cultured initially with CHX followed by addition of TNF-α and IL-6, no increase of detectable M-CSF mRNA levels was observed, demonstrating that for induction of M-CSF transcripts by these compounds new protein synthesis was required since the cytokines did not induce M-CSF gene expression above the CHX control. Phorbolesters such as TPA were also able to increase levels of M-CSF mRNA in fibroblasts after 4 h of culture (Fig. 5). Since TPA activates PKC and signal transduction pathways of TNF-α and IL-6 are still poorly understood, we investigated involvement of PKC in signaling of both cytokines. To this end, fibroblasts were exposed (24 h) to high concentrations of TPA (24 nM) followed by restimulation of cells with either low dose TPA (4 nM), TNF-α or IL-6 for an additional 4 h. Exposure of fibroblasts to TPA (24 nM) for 24 h decreased protein kinase C activity by >85%

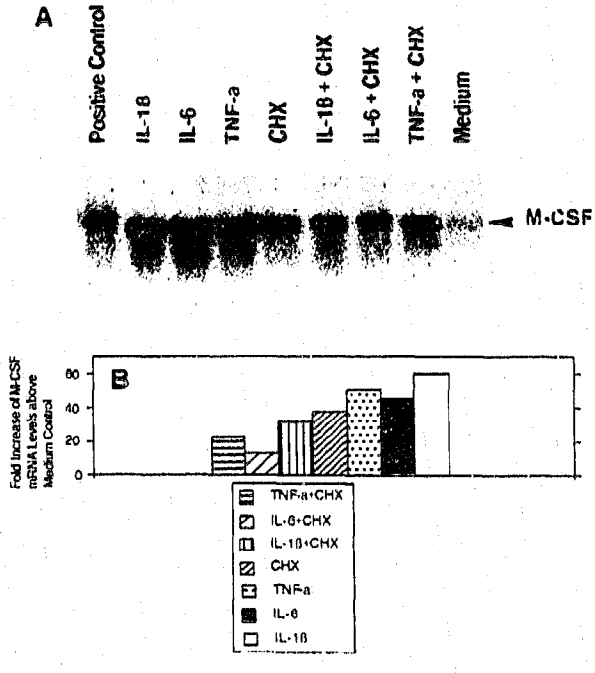

Fig. 4. Panel A; accumulation of M-CSF mRNA in fibroblasts cultured with IL-β, IL-6 and TNF-α in absence of new protein synthesis. Northern blot analysis (15 (μg) RNA per lane) of mRNA levels of M-CSF (4.0 kb) and β-actin (2.1 kb) in fibroblasts after initial culture for 30 min in the presence or absence of CHX (20 μg/ml). Cytokines were then added for a further 3 hrs alone with or without CHX (20 μg/ml). The concentrations of cytokines used correspond to those described in the legend to Fig. 1. The positive control represents mRNA derived from TPA (12 nM, 3 h) activated peripheral blood monocytes hybridized with the M-CSF specific cDNA. Panel B represents densitometric graphs (mean of two independent experiments with SD not exceeding 7%) of the same experiments as shown in panel A in which the data are normalized to actin mRNA levels.

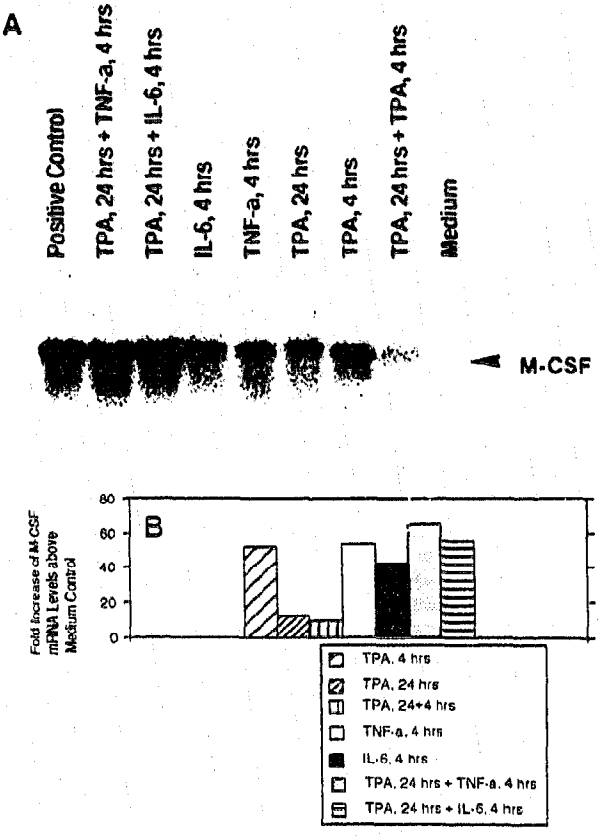

Fig. 5. Panel A: effect of prolonged exposure of phorbolester TPA on levels of M-CSF mRNA. Cells were pretreated with TPA (24 nM, 24 h) washed 3 times and were then exposed to rh IL-6 (10^3 U/ml), rh TNF-α (10^3 U/ml), TPA (8 nM) or medium alone for an additional 4 h. As control, fibroblasts were cultured for only 4 h with IL-6, TNF-α, TPA (concentrations as above) or medium only. The positive control represents mRNA derived from TPA (12 nM, 8 h) activated peripheral blood monocytes. Blots (20 μg RNA per lane) were hybridized sequentially with M-CSF (4.0 kb) and β-actin (2.1 kb) specific cDNA. Panel B represents densitometric graphs (mean of two independent experiments with SD not exceeding 7%) of the same experiments as shown in panel A in which the data are normalized to actin mRNA levels.

and decreased cell membrane-binding of [^{3}H]PDP by >95%, and re-exposure to TPA (4 nM) for 4 h did not stimulate the activity of protein kinase C (data not shown). As shown in Fig. 5, TPA precultured and thus PKC-depleted fibroblasts continued to be stimulated with TNF-α and IL-6, although being refractory to restimulation with TPA. Similarly, when TNF-α or IL-6 stimulated fibroblasts were exposed to the inhibitor of PKC H7, levels of M-CSF mRNA were comparable to those seen with TNF-α or IL-6 stimulation alone (Fig. 6). However, when culture of fibroblasts with TNF-α or IL-6 was preceeded by a 3 h exposure to H7, a dramatic increase of M-CSF mRNA levels (5.7-fold and 6-fold, respectively by densitometry analysis of autoradiographs) as compared to cultures simultaneously treated with H7 and TNF-α or IL-6 was observed. These results suggest the possibility that H7 could be inhibiting other protein kinases or that TNF-α and IL-6 do not utilize PKC-dependent signal pathways but that PKC may modulate receptiveness of fibroblasts to TNF-α and IL-6, and that inhibition of phosphorylation is associated with augmentation of the fibroblast response to both cytokines.

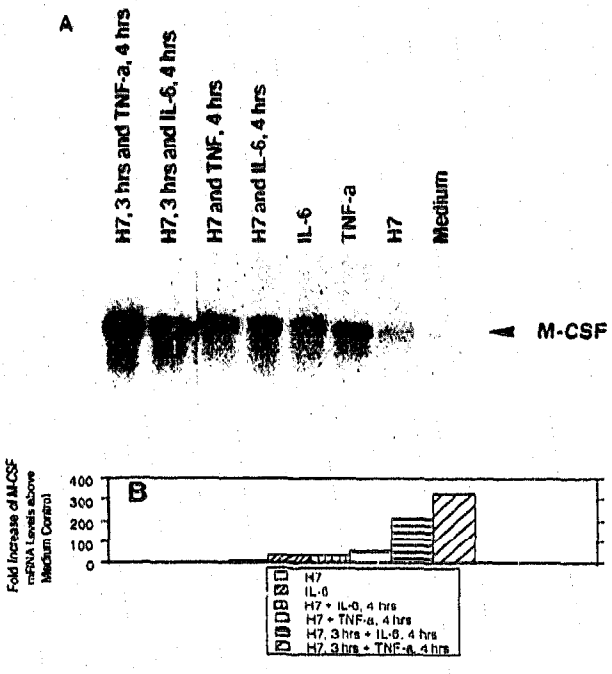

Fig. 6. Panel A: effect of inhibition of PKC on levels of M-CSF mRNA in fibroblasts cultured in the presence or absence of TNF-α and IL-6 (concentrations as indicated in the legend to Fig. 1). Fibroblasts were pretreated with the PKC inhibitor isoquinoline sulfonamide derivative H7 (30 μM; 3 h) washed 3 times and were then cultured with or without TNF-α or IL-6 for additional 4 h. In additional experiments fibroblasts were simultaneously exposed to H7 (30 μM) and TNF-α or IL-6 for a period of 4 h. In experiments not shown fibroblasts were treated with H7 (30 μM) and TNF-α or IL-6 for 7 h and still failed to modulate levels of M-CSF transcripts below or above those obtained from 7 h cultures with TNF-α or IL-6 only. Cytoplasmic RNA (10 μg per lane) was sequentially hybridized with M-CSF cDNA and β-actin cDNA. Panel B represents densitometric graphs (mean of two independent experiments with SD not exceeding 5%) of the same experiments as shown in panel A in which the data are normalized to actin mRNA levels.

4. DISCUSSION

In the present report we investigate the ability of various acute phase response cytokines including TNF-α, IL-1 β and IL-6 to regulate M-CSF expression in diploid human fibroblasts. In an unstimulated state, secretion of the M-CSF protein by fibroblasts is negligible although base-line transcription of the M-CSF gene and specific mRNA accumulation is detectable by nuclear run-on assay and Northern blot analysis. Exposure of fibroblasts to the 3 acute phase response cytokines resulted in detectable M-CSF protein in cell-free supernatants conditioned by fibroblasts. Mechanisms of action of these cytokines included both enhancement of the transcriptional rate of the M-CSF gene and increase of mRNA stabilization. It should be recognized, however, that serum which was used in all of the experiments may have exerted some of the effects noted. Particularly, it can not be excluded that some effects are indirect ones which may result from the interaction of cytokines with serum components. Our results show that the regulation of expression of M-CSF in fibroblasts differs from that previously reported in monocytes in which control of M-CSF levels takes place at the post-transcriptional level only [8,10]. Ongoing studies in our laboratory therefore address whether regulation of M-CSF expression is tissue-specific by using deletion vectors of the M-CSF promoter region. Since previous studies by others have shown that regulation of granulocyte/macrophage (GM)-CSF by acute phase response cytokines is independent of new protein synthesis [23], we determined the requirement for protein synthesis for induction of M-CSF mRNA accumulation by TNF-α and IL-6. We found that treatment of fibroblasts with the inhibitor of protein synthesis CHX (20 μg/ml) resulted in a significant enhancement of levels of M-CSF mRNA. When fibroblasts were precultured with CHX, subsequent culture with TNF-α or IL-6 failed to augment M-CSF mRNA levels above levels obtained with TNF-α or IL-6 alone. To induce M-CSF levels, TNF-α and IL-6 may therefore modify a preexisting protein that binds to regulatory sequences of the M-CSF gene. The transcription factor NF-$\varkappa$ B may be a candidate molecule in this regard [24]. To gain insights into TNF-α and IL-6 mediated signal transduction mechanisms leading to increased levels of M-CSF mRNA, an additional set of experiments was performed. Phorbolester TPA which acts through the PKC signaling pathway markedly increased M-CSF mRNA levels in fibroblasts. In contrast to earlier findings demonstrating that IL-1 β also utilizes the PKC pathway to induce cytokine expression [25], the ability of both TNF-α and IL-6 to induce M-CSF did not re-

quire PKC stimulation. It has been shown that prolonged exposure of fibroblasts to TPA (24 h) results in decrease of PKC activity by more than 80% in both cytosol and membrane fractions [14]. In our experiments re-exposure of fibroblasts to TPA, that were already pretreated with TPA for 24 h and thus depleted of PKC, failed to mount significant levels of M-CSF transcripts. However, TPA pretreated fibroblasts were not resistant to subsequent stimulation with both TNF-α and IL-6. This finding was consistent with data showing that simultaneous treatment of fibroblasts with TNF-α or IL-6 and PKC inhibitor H7 failed to alter levels of M-CSF detectable following treatment of fibroblasts with TNF-α or IL-6 alone. It has previously been shown that stability of certain mRNA species imparted by stability regulation sequences can be abrogated by PKC activation [26]. Inhibition of PKC may thus contribute to increased mRNA stability. Further studies will therefore be needed to determine synergy of H7 and TNF-α/IL-6 to prolong M-CSF mRNA decay. In this regard it has to be considered that M-CSF transcripts do not contain the typical AU-rich motif in their 3'untranslated region known to be involved in recognition and regulation of mRNA stability but contain several AU-rich stretches [27]. It has also been shown that PKC down-modulates expression of certain cytokine receptors such as TNF-α receptors [28]. PKC inhibition could thus contribute to augment ligand receptiveness. From a physiologic standpoint our data on M-CSF induction by acute phase response cytokines suggests that monocytes may augment their own number and function by establishing a paracrine loop involving secretion of acute phase response cytokines that stimulate local production of M-CSF by fibroblasts. Fibroblast-derived M-CSF could then turn on monocyte production and maintain their functional activity.

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